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(54) Title: GENE THERAPY FOR CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR ACTIVITY (CFTR)

(57) Abstract

Methods and compositions for producing a mammal capable of expressing an exogenously supplied gene in cells of the airway are disclosed. Lipid carrier-nucleic acid complexes or nucleic acid alone are prepared then delivered via aerosol or systemically to the lung alone or lung plus extrapulmonary tissues. The invention provides a direct method for transforming pulmonary cells as a means for treating the manifestations of CF in the lung and involved extrapulmonary tissues.

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GENE THERAPY FOR CYSTIC FIBROSIS TRANSMENBRANE CONDUCTANCE REGULATOR ACTIVITY (CFTR)

INTRODUCTION

Technical Field

The present invention relates to methods and compositions for producing a transgenic mammal which comprises exogenously supplied nucleic acid coding for a molecule having cystic fibrosis transmembrane conductance regulator activity. The nucleic acid is supplied by aerosolized delivery, particularly to the airways and alveoli of the lung, or by systemic delivery.

Background

Many genetic diseases are caused by the absence or mutation of the appropriate protein, for example as a result of deletions within the corresponding gene. One of the most common fatal genetic diseases in humans is cystic fibrosis (CF). Cystic fibrosis (CF), a spectrum of exocrine tissue dysfunction, which eventually leads to respiratory failure and death results from a mutation of the cystic fibrosis transmembrance conductance regulator (CFTR) gene. The CFTR gene has now been localized to chromosome 7q31, and cloned. A 3 bp deletion, resulting in the loss of a phenylalanine residue at amino acid position 508, is present in approximately 70% of CF chromosomes, but is not seen on normal chromosomes. The other 30% of CF mutations are heterogenous and include deletion, missense, and splice-site mutations. Transfection of even a single normal copy of the CFTR gene abolishes the CF secretory defect in CF cell lines, an observation which supports the feasibility of gene therapy for CF. These results demonstrate that expression of a wild-type CFTR transgene can exert a dominant positive effect in CF cells which concurrently express an endogenous mutant CFTR gene. Thus, expression of the wild-type CFTR transgene in the lungs of CF patients can correct the CF phenotype. However, to date, the inability to produce high level expression of transgenes in the lung by either aerosol or intravenous (iv) administration has precluded the use of gene therapy for the treatment of CF. Expression of a wild-type CFTR transgene in cells from CF patients corrects the chloride secretory defect, the primary biochemical lesion of CF. Chloride secretion is normalized in cells of CF patients despite the presence of the mutant CFTR

protein, indicating that when wild-type and mutant CFTR proteins are coexpressed in cells, the wild-type CFTR is dominant.

To date, attempts to replace absent or mutated genes in human patients have relied on *ex vivo* techniques. *Ex vivo* techniques include, but are not limited to, transformation of cells in vitro with either naked DNA or DNA encapsulated in liposomes, followed by introduction into a suitable host organ ("*ex vivo*" gene therapy). The criteria for a suitable organ include that the target organ for implantation is the site of the relevant disease, the disease is easily accessible, that it can be manipulated in vitro, that it is susceptible to genetic modification methods and ideally, it should contain either non-replicating cells or cycling stem cells to perpetuate a genetic correction. It also should be possible to reimplant the genetically modified cells into the organism in a functional and stable form. A further requirement for *ex vivo* gene therapy, if for example a retroviral vector is used, is that the cells be pre-mitotic; post-mitotic cells are refractory to infection with retroviral vectors. There are several drawbacks to *ex vivo* therapy. For example, if only differentiated, replicating cells are infected, the newly introduced gene function will be lost as those cells mature and die. *Ex vivo* approaches also can be used to transfect only a limited number of cells and cannot be used to transfect cells which are not first removed from the body. Exemplary of a target organ which meets the criteria of in vivo gene transfer is mammalian bone marrow; mammalian lung is not a good candidate for *ex vivo* therapy.

Retroviruses, adenoviruses and liposomes have been used in animal model studies in attempts to increase the efficiency of gene transfer. Liposomes have been used effectively to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcription factors and other cellular effectors into a variety of cultured cell lines and animals. In addition, successful clinical trials examining the effectiveness of liposome-mediated drug delivery have been completed. Several strategies have been devised to increase the effectiveness of liposome-mediated drug delivery by targeting liposomes to specific tissues and specific cell types. However, while the basic methodology for using liposome-mediated vectors is well developed, the technique has not been perfected for liposome-based transfection vectors for *in vivo* gene therapy. In the studies published to date, injection of the vectors either intravenously, intratracheally or into specific tissues has resulted in low but demonstrable expression, but the expression has generally been limited to one tissue, typically either the tissue that was injected (for example muscle); liver or lung where iv

injection has been used; or lung where intratracheal injection has been used, and less than 1% of all cells within these tissues were transfected.

In vivo expression of transgenes has been restricted to injection of transgenes directly into a specific tissue, such as direct intratracheal, intramuscular or intraarterial injection of naked DNA or of DNA-cationic liposome complexes, or to *ex vivo* transfection of host cells, with subsequent reinfusion. Currently available gene delivery strategies consistently have failed to produce a high level and/or generalized transgene expression *in vivo*. Expression of introduced genes, either complexed to cationic vectors or packaged in adenoviral vectors has been demonstrated in the lungs of rodents after intratracheal (IT) instillation. However, IT injection is invasive and produces a non-uniform distribution of the instilled material; it also is too invasive to be performed repeatedly in humans. For CF patients wherein the defect is a primary life-threatening defect in the lung, it would be of interest to develop a non-invasive delivery technique which also results in deeper penetration of exogenous nucleic acid constructs into the lung than do other methods, and can be used to deposit the CFTR gene constructs throughout the distal airways, as well as transfecting both airway epithelial cell and airway sub-mucosal cell types. Where other organs in the CF patient are affected due to the presence of mutant CFTR gene, techniques for transformation of a wide variety of tissues would be of interest, in order to alleviate extrapulmonary organ dysfunction in CF patients.

Relevant Literature

EP 91301819.8 (publication number 0 446 017 A1) discloses full length isolated DNAs encoding cystic fibrosis transmembrance conductance regulator (CFTR) protein and a variety of mutants thereof. Transient expression of CFTR in transformed cultured COS-7 cells is also disclosed. Rich *et al.*, *Nature* (1990) 347:358-363 and Gregory *et al.*, *Nature* (1990) 347:382-386 disclosed expression of the cystic fibrosis transmembrance conductance regulator in cultured HeLa cells using a vaccinia virus vector. Yoshimura *et al.* disclose expression of the CFTR gene in mouse lung after intratracheal administration of a plasmid containing the gene, either as naked DNA or complexed to lipofectin.

Brigham *et al.*, *Am. J. Med. Sci.* (1989) 298:278-281, describes the *in vivo* transfection of murine lungs with the CAT gene using a liposome vehicle. Transfection was accomplished by intravenous, intratracheal or intraperitoneal injection. Both intravenous and intratracheal administration resulted in the expression of the CAT gene in the lungs.

However, intraperitoneal administration did not. See, also Werthers, *Clinical Research* (1991) 39:(Abstract).

Canonico *et al.*, *Clin. Res.* (1991) 39:219A describes the expression of the human α-1 antitrypsin gene, driven by the CMV promoter, in cultured bovine lung epithelial cells. The gene was added to cells in culture using cationic liposomes. The experimenters also detected the presence of α-1 antitrypsin in histological sections of the lung of New Zealand white rabbits following the intravenous delivery of gene constructs complexed to liposomes. Yoshimura *et al.*disclose expression of the human cystic fibrosis transmembrane conductance regulator gene in mouse lung after intratracheal plasmid-mediated gene transfer.

Multiple approaches for introducing functional new genetic material into cells, both in vitro and *in vivo* have been attempted (Friedmann (1989) *Science*, 244:1275-1280). These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) *supra*; Rosenberg (1991) *Cancer Research* 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, *et al.* (1992) *Cell*, 68:143-155; Rosenfeld, *et al.* (1991) *Science*, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), *supra*; Brigham, *et al.* (1989) *Am. J. Med. Sci.*, 298:278-281; Nabel, *et al.* (1990) *Science*, 249:1285-1288; Hazinski, *et al.* (1991) *Am. J. Resp. Cell Molec.* Biol., 4:206-209; and Wang and Huang (1987) *Proc. Natl. Acad. Sci. (USA)*, 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) *J. Biol. Chem.*, 263:14621-14624) or the use of naked DNA expression vectors (Nabel *et al.* (1990), *supra*); Wolff *et al.* (1990) *Science*, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) *supra*); Rosenfeld *et al.* (1991) *supra*; Brigham *et al.* (1989) *supra*; Nabel (1990) *supra*; and Hazinski *et al.* (1991) supra). The Brigham *et al.* group (*Am. J. Med. Sci.* (1989) 298:278-281 and *Clinical Research* (1991) 39 (abstract)) have reported *in vivo* transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, *Science* (1992) 256:808-813.

PCT/US90/01515 (Felgner *et al.*) is directed to methods for delivering a gene coding for a pharmaceutical or immunogenic polypeptide to the interior of a cell of a vertebrate *in vivo*. Expression of the transgenes is limited to the tissue of injection. PCT/US90/05993 (Brigham) is directed to a method for obtaining expression of a transgene in mammalian lung cells following either iv or intratracheal injection of an expression

construct. PCT 89/02469 and PCT 90/06997 are directed to *ex vivo* gene therapy, which is limited to expressing a transgene in cells that can be taken out of the body such as lymphocytes. PCT 89/12109 is likewise directed to *ex vivo* gene therapy. PCT 90/12878 is directed to an enhancer which provides a high level of expression both in transformed cell lines and in transgenic mice using *ex vivo* transformation.

Debs *et al.* disclose pentamidine uptake in the lung by aerosolization and delivery in liposomes. *Am Rev Respir Dis* (1987) 135: 731-737. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger *et al.*, in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

SUMMARY

Methods and compositions are provided for producing a mammal which comprises exogenously supplied nucleic acid encoding a molecule having the biological activity of wild type cystic fibrosis transmembrane conductance regulator (CFTR) in its lung cells. The nucleic acid may be either a sense or an antisense strand of DNA. Also provided is a transgenic mammal comprising the CFTR nucleic acid. The method includes the steps of contacting host cells in vivo with a construct comprising said nucleic acid in an amount sufficient to transform cells contacted by the construct. The exogenously supplied nucleic acid generally is provided in a transcription cassette or an expression cassette and includes the coding sequence for a CFTR molecule operably joined to regulatory sequences functional in the mammal. The methods and compositions find use particularly for in vivo gene therapy of cystic fibrosis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows photomicrographs of frozen sections from lungs of control mice (Figures 1B and 1D) and mice treated with a plasmid containing the human CFTR gene (pZN32) complexed to DDAB:cholesterol (1:1) liposomes (Figures 1A, 1C, and 1E). Figures 1A, 1C, and 1E are lung sections from treated mice at 50X, 100X, and 250X magnification, respectively. Figures 1B and 1D are lung sections from untreated (control) mice at 50X and 100X magnification. Lipid carriers were 1 to 1 molar DDAB:Chol (SUV). Lipid carrier-DNA complexes were 5 nanomoles cationic lipid to 1 μg DNA. .

Figure 2A shows a section of mouse lung 48 hours following iv injection of PZN27:DDAB:Chol expression vector-cationic lipid carrier complexes. Lipid carrier composition was 1:1 molar DDAB:Chol. Lipid carrier plasmid ratio was 5 nanomoles cationic lipid to 1 μg DNA. A dose of 100 μg DNA was injected per mouse. This field shows alveoli and alveolar lining cells, the majority (50-70%) of which stain positively for the presence of CAT protein when probed with anti-CAT antibody and visualized using alkaline phosphatase. The treated animals' lungs stain uniformly with diffuse involvement of alveolar and vascular endothelial cells. Airway epithelial staining is also seen indicating airway are also transfected. The CAT (chloramphenicol acetyl transferase) protein normally is not present in mammalian cells and therefore the presence of CAT protein in these cells indicates that they have been transfected *in vivo*. Figure 2B shows a section of mouse lung from a control animal treated with iv-injected lipid carriers only, and probed with anti-CAT antibody. Cells do not show significant staining, although low-level background staining is detectable in some alveolar macrophages, which possess endogenous alkaline phosphatase activity.

Figure 3 shows construction of pZN20.

Figure 4 shows an electron micrograph which demonstrates that cationic lipid carrier: DNA complexes (DOTMA:DOPE:pRSV-CAT) are internalized by cells via classical receptor-mediated endocytosis following binding to cell surface receptors. Lipid carriers were 1:1 DOTMA:DOPE. 20 μg DNA was complexed with 20 nmoles cation lipid.

Figure 5 shows CAT gene expression in the indicated tissues following intravenous injection of pZN20:DDAB:DOPE complexes. Lipid carriers were DDAB:DOPE 1 to 1 molar. Two lipid carrier-to-plasmid ratios (nanomoles cationic lipid μg plasmid DNA) were used, MLV,6:1 and SUV, 3:1. Lanes 1-6 are samples from lung tissue; lanes 7-12, heart tissue; lanes 13-18, liver; lanes 19-24, kidney; lanes 25-30, spleen; lanes 31-36, lymph nodes. The first 3 samples of each tissue set were from animals injected with MLV, the next 3 samples of each tissue set were from animals injected with SUV. In lanes 1-18 the chromatograph runs from bottom to top, in lanes 19-36 the chromatograph runs from top to bottom. These results demonstrate that iv injection of pZN20:DDAB:DOPE complexes produces significant levels of CAT gene expression in six different tissues. Furthermore, MLV appear to mediate equal or greater levels of *in vivo* gene expression than do SUV composed of the same lipids.

Figure 6 shows the results of iv injection of DOTMA:DOPE complexed to pSIS-CAT plasmid does not produce detectable CAT expression *in vivo*. Figure 6A shows analysis of lung, spleen, liver and heart two days following iv injection with either lipid carrier alone (lanes 1-4) or lipid carrier plus DNA (lanes 5-8); Figure 6B shows the results at six days in mouse lung, spleen, liver and heart (lanes as in 6A. Lipid carriers were DOTMA:DOPE 1 to 1 molar. Cationic lipid to DNA ratio was 4 nanomoles to 1 μg. 100 μg DNA was injected per mouse. In both figures the chromatograph runs from bottom to top.

Figure 7 shows the construction of plasmid pZN27.

Figure 8 shows CAT gene expression in the indicated tissues following intravenous injection with pZN27 alone or pZN27:DDAB:Chol SUV complexes. Figure 8A lanes 1-10, lung; lanes 11-20, heart; lanes 21-30, liver; lanes 31-40, kidney; Figure 12B lanes 1-10, spleen; lanes 11-20, lymph nodes. Each tissue set of 10 contains samples treated with the following in order: 2 samples, 500 μg DNA; 2 samples, 1 mg DNA; 2 samples, 2 mg DNA; 2 samples, 500 μg DNA twice; 2 samples, lipid carrier-DNA complex 100 μg DNA. In Figure 8A lanes 1-20 the chromatograph runs from bottom to top; in Figure 8A lanes 21-40 the chromatograph runs from top to bottom. In Figure 8B lanes 1-20 the chromatograph runs from bottom to top. Lipid carriers were 1 to 1 molar DDAB:Chol. Lipid carrier-DNA complex was 5 nanomoles cationic lipid to 1 μg DNA.

Figure 9 shows CAT expression in the lung after intravenous injection of pRSV-CAT:L-PE:CEBA complexes. Lanes 1-3 are samples from untreated mouse lung, lane 4 is from a lung sample from a mouse treated with lipid carriers only, lane 5 is a sample from a mouse treated with the lipid carrier-DNA complex. Lipid carriers were 1 to 1 molar L-PE:CEBA. Lipid carrier-DNA complexes were 1 nanomole cationic lipid to 1 μg DNA. 100 μg DNA was injected per mouse. Chromatograph runs from bottom to top of Figure as shown.

Figure 10 shows the construction of plasmid pZN32.

Figure 11 shows the construction of plasmid pZN51.

Figure 12 shows the construction of plasmids pZN60, pZN61, pZN62 and pZN63. Figure 12A shows the construction of intermediate plasmids pZN52, pZN54, pZN56 and pZN58. Figure 12B shows the construction of the final plasmids, pZN60 through pZN63, from the intermediates.

Figure 13 shows an autoradiograph of the thin layer chromatograph of the CAT assay for six different plasmids injected intravenously in mice. Lanes 1-12 show the CAT activity

in lung tissue; Lanes 13-24 show the CAT activity in liver tissue. Lanes 1, 2, 13, 14-pZN51; lanes 3, 4, 15, 16-pZN60; lanes 5, 6, 17, 18-pZN61; lanes 7, 8, 19, 20-pZN62; lanes 9, 10, 21, 22-pZN63; lanes 11, 12, 23, 24-pZN27. Lipid carriers were DDAB:Chol (1:1). Lipid carriers-DNA complexes were 5nmoles cationic lipid to 1μg DNA. 100μg DNA was injected per mouse. Each lane represents a single mouse. Chromatograph runs from bottom to top of Figure as shown.

Figure 14(A-F) show CAT activity in heart (14A), spleen (14B), lung (14C), LN (14D), kidney (14E), and liner (14F) in lungs from uninjected mice (lanes 1-3), mice injected IV with pBE3.8CAT (lanes 4-6), or pCIS-CAT (lanes 7-9).

Figure 15 shows construction of pZN13.

Figure 16 shows construction of pZN29.

Figure 17 shows construction of pZN32.

Figure 19 shows a full restriction map for HCMV (Towne) of the immediate early enhancer and promoter region of HCMV (Towne) in Figure 19A and HCMV(AD169) in Figure 19C. Figure 19B shows a sequence comparison of the two HCMV promoters. The sequence of the Towne strain is designated as hs5miel on this comparison. The position of the *Nco*I site is indicated by an asterisk.

Figure 20 demonstrates that aerosol administration of pRSV-CAT-DOTMA: cholesterol complexes resulted in expression of the CAT gene in mouse lungs. Lanes 1-3 were derived from mice receiving no treatment; lanes 4-6 represent mice administered 0.5 mg pRSV-CAT with 1.0 μmole DOTMA-cholesterol liposomes; lanes 7-9 were derived from mice receiving 2.0 mg pRSV-CAT alone; and lanes 10-12 represent mice given 2.0 mg pRSV-CAT with 4.0 μmol DOTMA-cholesterol liposomes in a 2 to 1 molar ratio. The CAT gene is not normally present in mammalian cells; the results thus indicate that the lung was successfully transfected by the pRSV-CAT DOTMA-cholesterol:liposome aerosol. The results also show that neither aerosol administration of the pRSV-CAT alone, nor a lower aerosol dose of pRSV-CAT: DOTMA-cholesterol complexes produce detectable expression of the CAT gene in mouse lungs. Thus, both the cationic liposome carrier, and a sufficient dose of DOTMA: liposome complexes are required to produce transgene expression in the lung after aerosol administration, maximum transgene expression is achieved by complexing the liposomes and DNA together at an appropriate ratio dose and in an appropriate diluent.

Figure 21 shows the results of an experiment where mice were administered 12 mg of pCIS-CAT complexed to 24 μmoles of DOTMA/DOPE 1:1 liposomes. Lanes 1-3 show

the results from animals administered the aerosol in an Intox-designed nose-only aerosol exposure chamber; lanes 4-7 are derived from mice exposed to the aerosol in a modified mouse cage; and lanes 8-10 show the results from animals placed in a smaller modified cage after being put in restrainers originally constructed for use in the Intox chamber.

Figure 22 shows the results of immunostaining for intracellular CAT protein in lung sections from mice sacrificed 72 hours after receiving an aerosol containing 12 mg of pCIS-CAT plasmid complexed to 24 μmols of DOTMA:DOPE liposomes (A,B,C,D), or from untreated mice (E,F). The section shown in d was treated with normal rabbit serum in place of anti-CAT antibody. Magnification: A,D (x 50); B,C,E (x 250).

Figure 23 shows CAT activity in lung extracts from mice sacrificed 72 hours after receiving an aerosol containing either 12 mg of CMV-CAT plasmid alone or 12 mg of CMV-CAT plasmid complex to 24 μmols of DOTMA:DOPE (1:1) liposomes. Untreated mice were also assayed.

Figure 24 shows (A) CAT activity in lung extracts from mice sacrificed from one to twenty-one days after receiving an aerosol containing 12 mg of pCIS-CAT plasmid complexed to 24 μmols of DOTMA:DOPE liposomes; and (B) shows CAT activity in several different tissue extracts from mice and indicates that expression of the transgene is lung-specific after aerosolization of DNA-liposome complexes into normal mice sacrificed at the three day time point in Fig. 8A. Control extract contains CAT enzyme.

Figure 25 shows Southern blot hybridization of genomic DNA from the lungs of mice sacrificed immediately after receiving an aerosol containing 12 mg of pCIS-CAT plasmid complexed to 24 μmols of DOTMA:DOPE liposomes (lanes 1-4, 6-9) and from an untreated control mouse (lane 5). Samples were digested with the restriction enzyme *Hin*dIII and probed with a 1.6 kb CAT fragment (upper panel). The same membrane was hybridized with a 1.1 kb BSU 36-1 single copy probe from a mouse factor VIII.A genomic clone (lower panel).

Figure 26(A-F) shows histological analysis of CAT activity in lung from mice injected with CMV-CAT (Figs. 21A and 21D), CFTR-CAT (Figs. 21B and 21D) and centrol animals (Figs 21C and 21E).

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, nucleic acid constructs together with methods of preparation and use are provided which allow for *in vivo* modulation of phenotype and/or genotype of cells in the respiratory tract of a mammalian host following

delivery of a sufficient dose of a lipid carrier-nucleic acid aerosol to the host mammal or systemic delivery of a sufficient dose of nucleic acid, either naked or complexed with a lipid carrier. The nucleic acid is a nucleotide sequence which codes for a molecule having the biological activity of wild-type CFTR or it is a sequence which when transcribed provides an mRNA sequence complementary to the normal transcription product of an amount and/or of a size sufficient to block express of an endogenous CFTR gene, particularly a mutant CFTR gene. Of particular interest is expression of wild-type CFTR in lung airway cells as well as extrapulmonary cells which are dysfunctional in CF patients. Accordingly, the term "nucleic acid" as used herein refers to either the sense or the antisense strand coding for a molecule having CFTR activity. The lipid carrier-nucleic acid aerosol is obtained by nebulization of a lipid carrier-nucleic acid sample mixture prepared in a biologically compatible fluid that minimizes aggregation of the lipid carrier-nucleic acid complexes. The methods and compositions can be used to produce a mammal comprising an exogenously supplied nucleic acid coding for a molecule having CFTR activity in lung tissue, particularly airway passage cells, as well as submucosal cells, and appropriate exocrine cell types in non-pulmonary tissues.

Central to the present invention is the discovery that lung cells can be transfected via aerosol administration or systemic administration. The instant invention takes advantage of the use of lipid carriers as a delivery mechanism, although high doses of naked nucleic acid can also be used. Lipid carriers are able to stably bind nucleic acid through charge interactions so that resulting complexes may be nebulized and delivered to specific pulmonary tissues may be injected or using a nebulization device. Lipid carriers include but are not limited to liposomes and micelles, as well as biodegradable cationic compounds comprising modified phosphoglycerides, particularly alkylphosphaglycerides.

Lipid carriers, particularly liposomes, have been used effectively to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcription factors and other cellular effectors into a variety of cultured cell lines and animals. In addition, successful clinical trials examining the effectiveness of liposome-mediated delivery of small drug molecules and peptides which act extracellularly have been reported. However, while the basic methodology for using liposome-mediated vectors is well developed and has been shown to be safe, the technique previously has not been developed for delivery of nucleic acid to pulmonary tissue and appropriate extra-pulmonary tissues for *in vivo* gene therapy of genetic disorers related to mutant CFTR genes. By *in vivo* gene therapy is meant transcription

and/or translation of exogenously supplied nucleic acid to prevent, palliate and/or cure a disease or diseases related to mutant or absent CFTR genes and gene products.

Several factors have been identified that can affect the relative ability of lipid carrier-nucleic acid constructs to provide transfection of lung cells following aerosolized or systemic delivery of lipid carrier-nucleic acid constructs and to achieve a high level of expression. For aerosolized delivery, these factors include (1) preparation of a solution that prior to or during nebulization will not form macroaggregates and wherein the nucleic acid is not sheared into fragments and (2) preparation of both lipid carriers and expression constructs that provide for predictable transformation of host lung cells following aerosolization of the lipid carrier-nucleic acid complex and administration to the host animal. Other factors include the diluent used to prepare the solution and for either aerosolized or systemic delivery the lipidic vector:nucleic acid ratio solution for nebulization.

Aerosol delivery of nucleic acid-lipid carrier complexes provides a number of advantages over other modes of administration. For example, aerosol administration can serve to reduce host toxicity. Such an effect has been observed with the delivery of substances such as pentamidine and cytokines, which can be highly toxic when delivered systematically, but are well tolerated when aerosolized. See, for example, Debs *et al.*, *Antimicrob. Agents Chemother.* (1987) 31:37-41; Debs *et al.*, *Amer. Rev. Respir. Dis.* (1987) 135:731-737; Debs *et al.*, *J. Immunol.* (1988) 140:3482-3488; Montgomery *et al.*, *Lancet* (1987) 11:480-483; Montgomery *et al.*, *Chest* (1989) 95:747-751; Leoung *et al.*, *N. Eng. J. Med.* (1990) 323:769-775. Additionally, rapid clearance of circulating lipid carriers by the liver and spleen reticuloendothelial system is avoided, thereby allowing the sustained presence of the administered substance at the site of interest, the lung. Serum induced inactivation of the therapeutic agent is also reduced. This method of transfecting lung cells also avoids exposure of the host mammal's gonads, thus avoiding transfection of germ line cells.

Other advantages of the subject invention include ease of administration i.e., the host mammal simply inhales the aerosolized lipid carrier-nucleic acid solution into the intended tissue, the lung. Further, by varying the size of the nebulized particles some control may also be exercised over where in the lung the aerosol is delivered. Delivery may be extended over a long time period. Thus, there is a significant increase in the time period that target cells are exposed to the nucleic acid constructs. Distribution of the aerosol is even throughout areas of the lung accessible to the spray. These advantages are significant,

particularly when compared to other routes of administration such as intratracheal delivery which is invasive, the nucleic acid expression constructs are delivered in a bolus, which may disrupt the mucous barrier and additionally may result in pooling of the introduced fluid in areas of the lung at lower elevation. Damage from insertion of the intratracheal tube may alter the ability of cells coming into contact with the nucleic acid constructs to be transfected.

The type of vector used in the subject application also is an advantage over other available systems. For example, most gene therapy strategies have relied on transgene insertion into retroviral or DNA virus vectors. Potential disadvantages of retrovirus vectors, as compared to the use of lipid carriers, include the limited ability of retroviruses to mediate *in vivo* (as opposed to *ex vivo*) transgene expression; the inability of retrovirus vectors to transfect non-dividing cells; possible recombination events in replication-defect of retrovirus vectors, resulting an infectious retroviruses; possible activation of oncogenes or inhibition of tumor suppressor genes due to the random insertion of the transgene into host cell genomic DNA; size limitations (less than 15 kb of DNA can be packaged in a retrovirus vector, whereas lipid carriers can be used to deliver sequences of DNA of $\geq$ 250 kb to mammalian cells) and potential immunogenicity of the traditional vectors leading to a host immune response against the vector. In addition, all *ex vivo* approaches require that the cells removed from the body be maintained in culture for a period of time. While in culture, cells may undergo deleterious or potentially dangerous phenotypic and/or genotypic changes. Adenovirus and other DNA viral vectors share several of the above potential limitations. Particularly for human use, but also for repeated veterinary use, biodegradable lipid carriers, which are noninfectious, nonimmunogenic, and nonmutogenic may be used which are either metabolized or excreted by the host mammal to naturally occurring compounds that are non-toxic to the host and/or are readily excreted.

The constructs for use in the invention include several forms, depending upon the intended use of the construct. Thus, the constructs include vectors, trancriptional cassettes, expression cassettes and plasmids. The transcriptional and translational initiation region (also sometimes referred to as a "promoter,"), preferably comprises a transcriptional initiation regulatory region and a translational initiation regulatory region of untranslated 5′ sequences, "ribosome binding sites," responsible for binding mRNA to ribosomes and translational initiation. It is preferred that all of the transcriptional and translational functional elements of the initiation control region are derived from or obtainable from the

same gene. In some embodiments, the promoter will be modified by the addition of sequences, such as enhancers, or deletions of nonessential and/or undesired sequences. By "obtainable" is intended a promoter having a DNA sequence sufficiently similar to that of a native promoter to provide for the desired specificity of transcription of a DNA sequence of interest. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences.

The nucleic acid constructs generally will be provided as transcriptional cassettes. An intron optionally may be included in the construct, preferably $\geq$ 100 bp and placed 5′ to the coding sequence. Generally it is preferred that the construct not become integrated into the host cell genome and the construct is introduced into the host as part of a non-integrating expression cassette. A coding sequence is "operably linked to" or "under the control of" transcriptional regulatory regions in a cell when DNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, either a sense strand or an antisense strand. Thus, the nucleic acid sequence includes DNA sequences which encode polypeptides have the biological activity of CFTR which are directly or indirectly responsible for a therapeutic effect, as well as nucleotide sequences coding for nucleotide sequences such as antisense sequences and ribozymes.

In some cases, it may be desirable to use constructs that produce long-term effects *in vivo*, either by integration into host cell genomic DNA at high levels or by persistence of the transcription cassette in the nucleus of cells *in vivo* in stable, episomal form. Integration of the transcription cassette into genomic DNA of host cells *in vivo* may be facilitated by administering the transgene in a linearized form (either the coding region alone, or the coding region together with 5′ and 3′ regulatory sequences, but without any plasmid sequences present). Additionally, in some instances, it may be desirable to delete or inactivate a mutant CFTR gene and replace it with a coding sequence for a biologically functional CFTR molecule.

The constructs for use in the invention include several forms, depending upon the intended use of the construct. Thus, the constructs include vectors, trancriptional cassettes, expression cassettes and plasmids. The transcriptional and translational initiation region (also sometimes referred to as a "promoter,"), preferably comprises a transcriptional initiation regulatory region and a translational initiation regulatory region of untranslated 5′ sequences, "ribosome binding sites," responsible for binding mRNA to ribosomes and translational initiation. It is preferred that all of the transcriptional and translational

functional elements of the initiation control region are derived from or obtainable from the same gene. In some embodiments, the promoter will be modified by the addition of sequences, such as enhancers, or deletions of nonessential and/or undesired sequences. By "obtainable" is intended a promoter having a DNA sequence sufficiently similar to that of a native promoter to provide for the desired specificity of transcription of a DNA sequence of interest. It includes natural and synthetic sequences as well as sequences which may be a combination of synthetic and natural sequences.

For the transcriptional initiation region, or promoter element, any region may be used with the proviso that it provides the desired level of transcription of the DNA sequence of interest. The transcriptional initiation region may be native to or homologous to the host cell, and/or to the DNA sequence to be transcribed, or foreign or heterologous to the host cell and/or the DNA sequence to be transcribed. By foreign to the host cell is intended that the transcriptional initiation region is not found in the host into which the construct comprising the transcriptional initiation region is to be inserted. By foreign to the DNA sequence is intended a transcriptional initiation region that is not normally associated with the DNA sequence of interest. Efficient promoter elements for transcription initiation include the SV40 (simian virus 40) early promoter, the RSV (Rous sarcoma virus) promoter, the Adenovirus major late promoter, and the human CMV (cytomegalovirus) immediate early 1 promoter.

Inducible promoters also find use with the subject invention where it is desired to control the timing of transcription. Examples of promoters include those obtained from a β-interferon gene, a heat shock gene, a metallothionein gene or those obtained from steroid hormone-responsive genes, including insect genes such as that encoding the ecdysone receptor. Such inducible promoters can be used to regulate transcription of the transgene by the use of external stimuli such as interferon or glucocorticoids. Since the arrangement of eukaryotic promoter elements is highly flexible, combinations of constitutive and inducible elements also can be used. Tandem arrays of two or more inducible promoter elements may increase the level of induction above baseline levels of transcription which can be achieved when compared to the level of induction above baseline which can be achieved with a single inducible element.

Generally, the regulatory sequence comprises DNA up to about 1.5 Kb 5′ of the transcriptional start of a gene, but can be significantly smaller. This regulatory sequence may be modified at the position corresponding to the first codon of the desired protein by

site-directed mutagenesis (Kunkel TA, 1985, *Proc. Natl. Acad. Sci. (USA)*, 82:488-492) or by introduction of a convenient linker oligonucleotide by ligation, if a suitable restriction site is found near the N-terminal codon. In the ideal embodiment, a coding sequence with a compatible restriction site may be ligated at the position corresponding to codon #1 of the gene. This substitution may be inserted in such a way that it completely replaces the native coding sequence and thus the substituted sequence is flanked at its 3′ end by the gene terminator and polyadenylation signal.

Transcriptional enhancer elements optionally may be included in the expression cassette. By transcriptional enhancer elements is intended DNA sequences which are primary regulators of transcriptional activity and which can act to increase transcription from a promoter element, and generally do not have to be in the 5′ orientation with respect to the promoter in order to enhance transcriptional activity. The combination of promoter and enhancer element(s) used in a particular expression cassette can be selected by one skilled in the art to maximize specific effects. Different enhancer elements can be used to produce a desired level of transgene expression in a wide variety of tissue and cell types. For example, the human CMV immediate early promoter-enhancer element can be used to produce high level transgene expression in many different tissues *in vivo*.

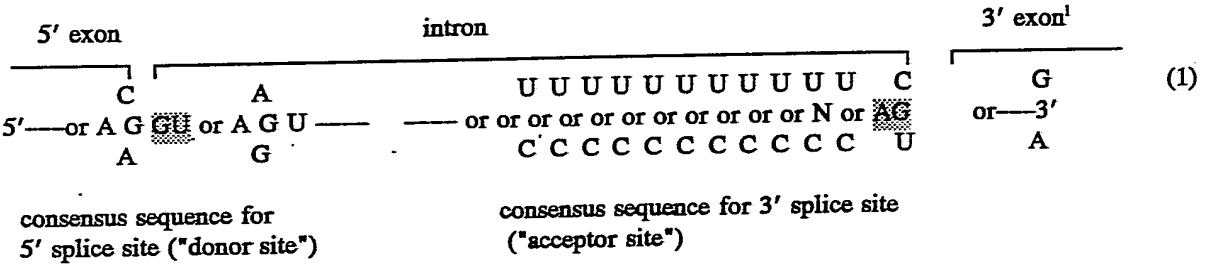
Examples of other enhancer elements which confer a high level of transcription on linked genes in a number of different cell types from many species include enhancers from SV40 and RSV-LTR. The SV40 and RSV-LTR are essentially constitutive. They may be combined with other enhancers which have specific effects, or the specific enhancers may be used alone. Thus, where specific control of transcription is desired, efficient enhancer elements that are active only in a tissue-, developmental-, or cell-specific fashion include immunoglobulin, interleukin-2 (IL-2) and ß-globin enhancers are of interest. Tissue-, developmental-, or cell-specific enhancers can be used to obtain transgene expression in particular cell types, such as B-lymphocytes and T-lymphocytes, as well as myeloid, or erythroid progenitor cells. Alternatively, a tissue-specific promoter such as that derived from the human cystic fibrosis transmembrane conductance regulator (CFTR) gene can be fused to a very active, heterologous enhancer element, such as the SV40 enhancer, in order to confer both a high level of transcription and tissue-specific transgene transcription. In addition, the use of tissue-specific promoters, such as LCK, may allow targeting of transgene transcription to T lymphocytes. Tissue specific transcription of the transgene may

be important, particularly in cases where the results of transcription of the transgene in tissues other than the target tissue would be deleterious.

Tandem repeats of two or more enhancer elements or combinations of enhancer elements may significantly increase transgene expression when compared to the use of a single copy of an enhancer element; hence enhancer elements find use in the expression cassette. The use of two different enhancer elements from the same or different sources flanking or within a single promoter can in some cases produce transgene expression in each tissue in which each individual enhancer acting alone would have an effect, thereby increasing the number of tissues in which transcription is obtained. In other cases, the presence of two different enhancer elements results in silencing of the enhancer effects. Evaluation of particular combinations of enhancer elements for a particular desired effect or tissue of expression is within the level of skill in the art.

Although generally it is not necessary to include an intron in the expression cassette, an intron comprising a 5′ splice site (donor site) and a 3′ splice site (acceptor site) separated by a sufficient intervening sequence to produce high level, extended *in vivo* expression of a transgene administered iv or ip can optionally be included. Generally, an intervening sequence of about 100bp produces the desired expression pattern and/or level, but the size of the sequence can be varied as needed to achieve a desired result. The optional intron placed 5' to the coding sequence results in high level extended *in vivo* expression of a transgene administered iv or ip but generally is not necessary to obtain expression. Optimally, the 5' intron specifically lacks cryptic splice sites which result in aberrantly spliced mRNA sequences. If used, the intron splice donor and splice acceptor sites, arranged from 5′ to 3′ respectively, are placed between the transcription initiation site and the translational start codon as diagrammed in (1), below.

Consensus sequences for the 5′ and 3′ splice sites used in RNA splicing



[1] The sequence given is that for the RNA chain; the nearly invariant GU and AG dinucleotides at either end of the intron are shaded.

Alternatively, the intervening sequence may be placed 3′ to the translational stop codon and the transcriptional terminator or inside the coding region. The intron can be a hybrid intron with an intervening sequence or an intron taken from a genomic coding sequence. An intron 3' to the coding region, a 5' intron which is of less than 100 bp, or an intron which contains cryptic splice sites may under certain condition substantially reduce the level of transgene expression produced *in vivo*. However, unexpectedly, a high level of *in vivo* expression of a transgene can be achieved using a vector that lacks an intron. Such vectors therefore are of particular interest for *in vivo* transfection.

Downstream from and under control of the transcriptional initiation regulatory regions is a multiple cloning site for insertion of a nucleic acid sequence of interest which will provide for one or more alterations of host genotype and modulation of host phenotype. Conveniently, the multiple cloning site may be employed for a variety of nucleic acid sequences in an efficient manner. The nucleic acid sequence inserted in the cloning site may have any open reading frame encoding a polypeptide of interest, for example, an enzyme, with the proviso that where the coding sequence encodes a polypeptide of interest, it should lack cruptic splice sites which can block production of appropriate mRNA molecules and/or produce aberrantly spliced or abnormal mRNA molecules. The nucleic acid sequence may be DNA; it also may be a sequence complementary to a genomic sequence, where the genomic sequence may be one or more of an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, for example splicing, or translation.

The incidence of integration of the transcription cassette into genomic DNA may be increased by incorporating a purified retroviral enzyme, such as the HIV-1 integrase enzyme, into the lipid carrier-DNA complex. Appropriate flanking sequences are placed at the 5′ and 3′ ends of the nucleic acid. These flanking sequences have been shown to mediate integration of the HIV-1 DNA into host cell genomic DNA in the presence of HIV-1 integrase. Alternatively, the duration of the expression of the exogenous nucleic acid *in vivo* can be prolonged by the use of constructs that contain non-transforming sequences of a virus such as Epstein-Barr virus, and sequences such as oriP and EBNA-1 which appear to be sufficient to allow heterologous DNA to be replicated as an episome in mammalian cells (Buhans *et al.*, Cell (1986) 52:955).

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region

may be native to the intended nucleic acid sequence of interest, or may be derived from another source. Convenient termination regions are available and include the 3′ end of a gene terminator and polyadenylation signal from the same gene from which the 5′ regulatory region is obtained. Adenylation residues, preferably more than 32 and up to 200 or more as necessary may be included in order to stabilize the mRNA. Alternatively, a terminator and polyadenylation signal from different gene/genes may be employed with similar results. Specific sequences which regulate post-transcriptional mRNA stability may optionally be included. For example, certain polyA sequences (Volloch *et al. Cell* (1981) 23:509) and ß-globin mRNA elements can increase mRNA stability, whereas certain AU-rich sequences in mRNA can decrease mRNA stability (Shyu *et al.*, Genes and Devel. (1989) 3:60). In addition, AU regions in 3′ non-coding regions may be used to destabilize mRNA if a short half-life mRNA is desirable for the gene of interest.

Isolation of Genes and Construction of Vectors

Nucleic acid sequences for use in the present invention, can be derived from known sources, for example by isolating the nucleic acid from cells containing the desired gene, using standard techniques. Similarly, the gene sequence can be generated synthetically, using standard modes of polynucleotide synthesis, well known in the art. See, e.g. Edge, M.D., *Nature* (1981) 292:756; Nambair, *et al.*, *Science* (1984) 223:1299; Jay, Ernest, J Biol Chem (1984) 259:6311. Generally, synthetic oligonucleotides are prepared by either the phosphotriester method as described by Edge *et al.*, *Nature* (supra) and Duckworth *et al.*, *Nucleic Acids Res* (1981)9:1691, or the phosphoramidite method as described by Beaucage, S.L., and Caruthers, M.H., *Tet. Letts.* (1981) 22:1859, and Matteucci, M.D., and Caruthers, M.H., *J. Am. Chem. Soc.* (1981) 103:3185, and can be prepared using commercially available automated oligonucleotide synthesizers. The gene sequence can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for expression in the intended host. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair *et al.*, (1984) *Science* 223:1299; Jay *et al.*, (1984) *J. Biol. Chem.* 259:6311. Partial CFTR cDNA clones T11 T16-1 T16-4.5 and C1-1/5 (Riordan *et al.*, *Science* (1989) 245:1066-1073) are available from the American Type Culture Collection (Rockland, Maryland). Full length isolated DNAs encoding CFTR protein and a variety of

mutants thereof are disclosed in EP Application 91301819.8. See also, Goodfellow, P., *Nature* (1989) 341:102-103; Rommens, *et al.*, *Science* (1989) 245:1059-1054; Beardsley, *et al.*, *Sci. Am.* (1989) 261:28-30. It may be desirable to produce mutants or analogs of the proteins of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. The mutation can be one that affects secretion of a normally secreted protein, so as to eliminate or decrease systemic side effects of the protein. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., Sambrook *et al.*, infra; *DNA Cloning*, Vols. I and II, supra; *Nucleic Acid Hybridization*, infra.

A particularly convenient method for obtaining nucleic acid for use in the lipid carrier-nucleic acid preparations, is by recombinant means. Thus, the CFTR gene can be excised from a plasmid carrying the desired gene, using standard restriction enzymes and procedures. Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1950) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using standard techniques. The Klenow fragment fills in at 5′ single-stranded overhangs but chews back protruding 3′ single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the overhang. After treatment with Klenow, the mixture can be extracted with e.g. phenol/chloroform, and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or BAL-31 results in hydrolysis of any single-stranded portion.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art; the selection of an appropriate cloning vector is known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of

choice. Ligation to other sequences is performed using standard procedures, known in the art. For example, ligations can be accomplished in 20 mM Tris-C1 pH 7.5, 10 mM $MgC1_2$, 10mM DTT, 33 μg/ml BSA, 10 mM-50 mM NaC1, and either 40 uM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 30-100 μg/ml total DNA concentration (5-100 nM total end concentration).

The nucleic sequence is placed under the control of a promoter, ribosome binding site and, optionally, an operator (collectively referred to herein as "control" elements), so that the coding sequence is transcribed into RNA in the host tissue transformed by the lipid carrier-nucleic acid. The coding sequence may or may not contain a signal peptide or leader sequence. A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3′ terminus by the transcription start codon (ATG) of a coding sequence and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence is a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Nucleic acid "control sequences" or "regulatory regions" refer collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

The choice of regulatory elements will depend on the host cell which is to be transformed and the type of nucleic acid preparation used. Thus, if the host cells' endogenous transcription and translation machinery will be used to express a CFTR molecule, control elements functional in the particular host and which provide for expression are used. Several promoters for use in mammalian cells are known in the art and include, but are not limited to, a SV40 (Simian Virus 40) early promoter, a RSV (Rous Sarcoma Virus) promoter, an Adenovirus major late promoter, and a human CMV (Cytomegalovirus) immediate early one promoter. Other promoters which may be used include those derived from mouse mammary tumor virus (MMTV, T7, T3, and the like). Particularly useful in

the present invention are the RSV promoter and the CMV promoter, particularly the immediate early promoter from the AD169 strain of CMV. In addition to the above sequences, it may be desirable to add to the nucleic acid construct regulatory sequences which allow for regulation of the expression of the CFTR molecule. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Such promoters can be used to regulate expression of the transgene by the use of external stimuli such as interferon or glucocorticoids.

Other types of regulatory elements may also be present in the plasmid, for example, enhancer sequences. Such regulatory elements include those obtainable from β-interferon, heat shock, metallothionein or steroid hormone responsive genes, including insect genes such as the ecdysone receptor gene. Since the arrangement of eukaryotic promoter elements is highly flexible, combinations of constitutive and inducible elements can be used. Tandem arrays of two or more inducible promoter elements may increase the level of induction above baseline levels of transcription which can be achieved with a single inducible element. By transcription enhancer elements are intended DNA sequences which are primary regulators of transcriptional activity which can act to increase transcription from a promoter element, and generally do not have to be in the 5′ orientation with respect to the promoter in order to enhance transcriptional activity.

The combination of promoter and enhancer elements used in a particular nucleic acid construct can be selected by one skilled in the art to maximize specific effects; different enhancer elements can be used to produce a desired level of transcription. For example, a tissue specific promoter such as that derived from the human cystic fibrosis transmembrane conductance regulator (CFTR) gene can be used flanking a very active, heterologous enhancer element, such as the SV40 enhancer, in order to obtain both a high level of expression and expression of the nucleic acid primarily in lung. Tandem repeats of two or more enhancer elements or combinations of enhancer elements may significantly increase transcription when compared to the use of a single copy of an enhancer element. The use of two different enhancer elements from the same or different sources, flanking or within a single promoter may be used. Evaluation of particular combinations of enhancer elements for a particular desired effect or expression level is within the knowledge of one skilled in the art. Promoter-enhancer elements which are least partially derived from CMV

Townes and/or AD169 strains are of particular interest for providing a high level of expression of exogenous nucleic acid.

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region may be native to the CFTR gene, or may be derived from another source. Convenient termination regions are available and include the 3′ end of a gene terminator and polyadenylation signal from the same gene from which the 5′ regulatory region is obtained. Adenylation residues, preferably more than 32 kb and up to 200 kb or more if necessary may be included in order to stabilize the mRNA. Alternatively, terminator and polydenylation signals from a gene/genes other than the CFTR gene may be employed with similar results. Specific sequences which regulate post-transcriptional mRNA stability may optionally be included. For example, certain polyA sequences (Volloch *et al.*, Cell (1981) 23:509) and β-globin mRNA elements can increase mRNA stability, whereas certain AU-rich sequences in mRNA can decrease mRNA stability (Shyu *et al.*, Genes and Development (1989) 3:60). In addition, AU regions in 3′ non-coding regions may be used to destabilize mRNA if a short half life mRNA is desirable. A 3′-intron should be avoided, particularly a SV40 3′-intron. If used, the 3′-intron should be greater than about 70 bp.

The nucleic acid construct may include sequences for selection, such as a neomycin resistance gene, dihydrofolate reductase gene, and/or signal sequences to generate recombinant proteins that are targeted to different cellular compartment, more particularly to provide for secretion of the nucleic acid expression product. Any of a variety of signal sequences may be used which are well known to those skilled in the art, for example, a basic sequence of amino acids may be encoded which results in nucleic localization? of the protein.

A transcription vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences. Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be

cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

Preparation of Lipid carriers

Lipid carriers for use in the instant invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. However, cationic lipid carriers are particularly preferred because a tight charge complex can be formed between the cationic lipid carrier and the polyanionic nucleic acid. For example, this results in a lipid carrier-nucleic acid complex which will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells after the aerosolized DNA:lipid carrier complex has been deposited in the lung. Cationic lipid carriers have been shown to mediate intracellular delivery of plasmid DNA (Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416); mRNA (Malone, *et al.*, *Proc. Natl. Acad. Sci. USA* (1989) 86:6077-6081); and purified transcription factors (Debs, *et al.*, *J. Biol. Chem.* (1990) 265:10189-10192), in functional form.

Particular cells within the lung and extrapulmonary organs may be targeted by modifying the lipid carriers to direct them to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors may be employed, to target a cell associated with a particular surface protein. A particular ligand or antibody may be conjugated to the lipid carrier in accordance with conventional ways, either by conjugating the site-directing molecule to a lipid for incorporation into the lipid bilayer or by providing for a linking group on a lipid present in the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art. Precise intrapulmonary targeting also may be achieved by a) altering aerosol particle size to preferentially direct the aerosol to alveoli or proximal versus distal airways or (b) to covalently couple monoclonal antibodies to the lipid carrier surface, thereby targeting lung cells expressing the corresponding cell surface antigen.

The various lipid carrier-nucleic acid complexes wherein the lipid carrier is a liposome are prepared using methods well known in the art. See, e.g., Straubinger *et al.*, in *Methods of Immunology* (1983), Vol. 101, pp. 512-527. By "lipid carrier-nucleic acid complex" is meant a nucleic acid sequence as described above, generally bound to the surface of a lipid carrier preparation, as discussed below. The lipid carrier preparation can

also contain other substances, such as enzymes necessary for integration, transcription and translation, cofactors, etc. Furthermore, the lipid carrier-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types. Where it is desired to entrap the nucleic acid, MLVs containing nucleic acid can be prepared by depositing a thin firm of phospholipid on the walls of a glass tube and subsequently hydrating with a solution of the material to be encapsulated and vortexing.

The nucleic acid material is added to a suspension of preformed MLVs or SLVs only after the lipid carriers have been prepared and then vortexed. When using lipid carriers containing cationic lipids, the dried lipid film is resuspended in an appropriate mixing solution such as sterile water or an isotonic buffer solution such as 10mM Tris/NaCl, or 5% dextrose in sterile water, sonicated, and then the preformed lipid carriers are mixed directly with the DNA. The lipid carrier and DNA form a very stable complex due to binding of the negatively charged DNA to the cationic lipid carriers. SUVs find use with small nucleic acid fragments as well as large regions of DNA ($\geq$ 250 kb).

In preparing the lipid carrier-nucleic acid complex, care should be taken to exclude any compounds from the mixing solution which may promote the formation of aggregates of the lipid carrier-nucleic acid complexes. For aerosol administration, large particles generally will not be aerosolized by the nebulizer and even if aerosolized would be too large to penetrate beyond the large airways. Aggregation of the lipid carrier-nucleic acid complex is prevented by controlling the ratio of DNA to lipid carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution, usually less than 5 mg DNA/8 ml solution, and the avoiding chelating agents as EDTA, and significant amounts of salt which tend to promote macroaggregation. The preferred excipient is water, dextrose/water or another solution having low or no ionic strength. Further, the volume must be adjusted to the minimum for deposition in the lungs of the host mammal, but taking care not to make the solution too concentrated so that aggregates form.

The choice of lipid carriers and the concentration of lipid carrier-nucleic acid complexes thus involves a two step process. The first step is to identify lipid carriers and concentration of lipid carrier-nucleic acid complexes that do not aggregate when the components are combined or during the significant agitation of the mixture that occurs during the nebulization step. The second step is to identify among those that are identified as of interest at the first step (i.e. do not aggregate) those complexes that provide for a high level of transfection and expression of a gene of interest in target cells in the lung. The

level of expression and the cell types in which expression of the recombinant gene is obtained may be determined at the mRNA level and/or at the level of polypeptide or protein. Gene product may be quantitated by measuring its biological activity in tissues. For example, enzymatic activity can be measured by biological assay or by identifying the gene product in transfected cells by immunostaining techniques such as probing with an antibody which specifically recognizes the gene product or a reported gene product present in the expression cassette.

As an example, a reporter gene CAT (which encodes chloramphenicol acetyl transferase) can be inserted in the expression cassette and used to evaluate each lipid carrier composition of interest. The DNA:lipid carrier complexes must be mixed in solutions which do not themselves induce aggregation of the DNA:lipid carrier complexes such as sterile water. The expression cassette (DNA) is mixed together with the lipid carriers to be tested in multiple different ratios, ranging as an example from 4:1 to 1:10 (micrograms DNA to nanomoles cationic lipid). The results provide information concerning which ratios result in aggregation of the DNA:lipid carrier complexes and are therefore not useful for use in vivo, and which complexes remain in a form suitable for aerosolization. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confer the highest level of transgene expression *in vivo*. For example, the optimal DNA:lipid carrier ratios for SUV for DOTMA/DOPE DDAB:Chol, are 1:1 or 1:2 and ethylphosphatidylcholine (E-PC and ethyl-dimyristylphosphatidylcholine (E-DMPC).

Aerosol Administration

The mammalian host may be any mammal having symptoms of a genetically-based disorder. Thus, the subject application finds use in domestic animals, feed stock, such as bovine, ovine, and porcine, as well as primates, particularly humans. In the method of the invention, transformation *in vivo* is obtained by introducing a non-integrating therapeutic plasmid into the mammalian host complexed to a lipid carrier, particularly a cationic lipid carrier more particularly, for human use or for repeated applications a biodegradable lipid carrier. For introduction into the mammalian host any physiologically acceptable medium may be employed for administering the DNA or lipid carriers, such as deionized water, 5% dextrose in water, and the like. Other components may be included in the formulation such as stabilizers, biocides, etc, providing that they meet the criteria outlined above, i.e. do not

cause aggregation of the complexes. The various components listed above find extensive exemplification in the literature and need not be described in particular here.

For aerosol delivery in humans or other primates, the aerosol is generated by a medical nebulizer system which delivers the aerosol through a mouthpiece, facemask, etc. from which the mammalian host can draw the aerosol into the lungs. Various nebulizers are known in the art and can be used in the method of the present invention. See, for example, Boiarski, *et al.*, U.S. Patent No. 4,268,460; Lehmbeck, *et al.*, U.S. Patent No. 4,253,468; U.S. Patent No. 4,046,146; Havstad, *et al.*, U.S. Patent No. 3,826,255; Knight, *et al.*, U.S. Patent No. 4,649,911; Bordoni, *et al.*, U.S. Patent No. 4,510,829. The selection of a nebulizer system depends on whether alveolar or airway delivery (i.e., trachea, primary, secondary or tertiary bronchi, etc.), is desired.

A convenient way to insure effective delivery of the nucleic acid to the alveoli is to select a nebulizer which produces sufficiently small particles for example, particles with a mean particle diameter of less than 5.0 microns (μm). More preferably the particles have a mean particle diameter of about 0.2 to about 4.0 μm, and most preferably the particles have mean diameter of about 0.2 to about 2 μm, since larger particles ($\geq$ 5 μm) are generally deposited in the proximal airways or nasopharynx. As an alternative to selecting small mean particle diameters to achieve substantial alveoli deposition, a very high dosage of the lipid carrier-nucleic acid preparation can be administered, with a larger mean particle diameter. A proviso to such an approach is that the particular lipid carrier-nucleic acid complex is chosen that is not too irritating at the required dosage and that there be a sufficient number of particles in the total particle population having a diameter in the 0.5 to about 5 μm range to allow for deposition in the alveoli. For proximal airway delivery, the mean particle size will be larger. For example, suitable mean particle diameters will generally be less than about 15 μm, more preferably from about 4 μm, and most preferably from about 5 μm to about 10 μm.

Examples of nebulizers useful for alveolar delivery include the Acorn 1 nebulizer, and the Respirgard II® Nebulizer System, both available commercially from Marquest Medical Products, Inc., Inglewood, CO. Other commercially available nebulizers for use with the instant invention include the UltraVent® nebulizer available from Mallinckrodt, Inc. (Maryland Heights, MO); the Wright nebulizer (Wright, B.M., *Lancet* (1958) 3:24-25); and the DeVilbiss nebulizer (Mercer *et al.*, *Am. Ind. Hyg. Assoc. J.* (1968) 29:66-78; T.T. Mercer, *Chest* (1981) 80:6 (Sup) 813-817). Nebulizers useful for airway

delivery include those typically used in the treatment of asthma. Such nebulizers are also commercially available. One of skill in the art can determine the usefulness of a particular nebulizer by measuring the mean particle size generated thereby with for example, a 7 stage Mercer cascade impactor (Intox Products, Albuquerque, NM). Concentrations of the lipid carrier-nucleic acid complex from the impactor plates can be determined by eluting the complex therefrom and assessing the optical density at an appropriate wavelength and comparing the standard curves. Results are generally expressed as mass median aerodynamic diameter ± geometric standard deviation (Raabe, *J. Aerosol Sci.* (1971) 2:289-303).

The amount of lipid carriers used will be an amount sufficient to provide for adequate transfection of cells after entry of the DNA or complexes into the lung and to provide for a therapeutic level of transcription and/or translation in transfected cells. A therapeutic level of transcription and/or translation is a sufficient amount to prevent, treat, or palliate a disease of the host mammal following administration of the lipid carrier-nucleic acid complex to the host mammal's lung, particularly the alveoli or airway. Thus, an "effective amount" of the aerosolized lipid carrier-nucleic acid preparation, is a dose sufficient to effect treatment, that is, to cause alleviation or reduction of symptoms, to inhibit the worsening of symptoms, to prevent the onset of symptoms, and the like. The dosages of the present compositions which constitute an effective amount can be determined in view of this disclosure by one of ordinary skill in the art by running routine trials with appropriate controls. Comparison of the appropriate treatment groups to the controls will indicate whether a particular dosage is effective in preventing or reducing particular symptoms. Appropriate doses are discussed further below. While there is no direct method of measuring the actual amount of lipid carrier-nucleic acid complex delivered to the alveoli, bronchoalveolar lavage (BAL) can be used to indirectly measure alveolar concentrations of any expressed and secreted protein, usually 18-24 hrs after inhalation to allow clearance of the protein deposited in the larger airways and bronchi.

The total amount of nucleic acid delivered to a mammalian host will depend upon many factors, including the total amount aerosolized, the type of nebulizer, the particle size, breathing patterns of the mammalian host, severity of lung disease, concentration and mean diameter of the lipid carrier-nucleic acid complex in the aerosolized solution, and length of inhalation therapy. Thus, the amount of expressed protein measured in the airways may be substantially less than what would be expected to be expressed from the amount of

nucleic acid present in the aerosol, since a large portion of the complex may be exhaled by the subject or trapped on the interior surfaces of the nebulizer apparatus. For example, approximately one third of the lipid carrier-nucleic acid dose that is placed into the nebulizer remains in the nebulizer and associated tubing after inhalation is completed. This is true regardless of the dose size, duration of inhalation, and type of nebulizer used. Moreover, resuspension of the residue and readministration does not significantly increase the dose delivered to the subject; about one third remains in the nebulizer. Additionally, efficiency of expression of the encoded protein will vary widely with the expression system used.

Despite the interacting factors described above, one of ordinary skill in the art will be able readily to design effective protocols, particularly if the particle size of the aerosol is optimized. Based on estimates of nebulizer efficiency, an effective dose delivered usually lies in the range of about 1 mg/treatment to about 500 mg/treatment, although more or less may be found to be effective depending on the subject and desired result. It is generally desirable to administer higher doses when treating more severe conditions. Generally, the nucleic acid is not integrated into the host cell genome, thus if necessary, the treatment can be repeated on an ad hoc basis depending upon the results achieved. If the treatment is repeated, the mammalian host is monitored to ensure that there is no adverse immune response to the treatment. The frequency of treatments depends upon a number of factors, such as the amount of lipid carrier-nucleic acid complex administered per dose, as well as the health and history of the subject. As used herein, with reference to dosages, "lipid carrier-nucleic acid aerosol" refers to the amount of lipid carrier-nucleic acid complex that is placed in the nebulizer and subjected to aerosolization. The "amount nebulized" or "amount aerosolized" of the complex means the amount that actually leaves the apparatus as an aerosol, i.e., the amount placed into the apparatus less the amount retained in the reservoir and on the inner surfaces of the apparatus at the conclusion of a treatment session.

To treat pulmonary infections such as bronchitis and pneumonia, it will usually be necessary to administer at least one dose per day over a period of about 4 to about 21 consecutive days or longer. The treatment is usually carried out on consecutive days because new areas of the lungs open up to penetration and deposition of the nucleic acid with increasing resolution of the infection. The success of the treatment can be monitored and the administration regimen altered by assessing conventional clinical criteria; e.g., clearing of radiographic infiltrate, improved arterial PO_2 (e.g., >70 mmHg), reduction in dyspnea, respiratory rate and/or fever. For the treatment of genetic disorders, such as

cystic fibrosis, the lipid carrier-nucleic acid complex will be administered at regular intervals, from once a week to once every one to several months, in order to replace the normal CRTR protein in critical host airway cells, since these cells continue to turn over. It may also be possible to stably transfect the CFTR gene into appropriate lung stem cells, which would then provide a continuous source of normal airway cells without requiring lifelong treatment. Potential therapeutic effects of the gene product can be measured, by determining the effects of gene expression on survival of transgenic host mammals in which the transgene is expressed. Production of significant amounts of a transgene product will substantially prolong the survival and improve the quality of life of the afflicted host.

Where expression of the polypeptide/protein or even the mRNA itself confers a changed biochemical phenotype upon the host, the presence of a new phenotype or absence of an old phenotype may be evaluated; for example, as a result of transformation of the host cells, there may be enhanced production of pre-existing desirable products formerly produced in insufficient quantities or there may be reduction or even suppression of an undesirable gene product using antisense, ribozyme or co-suppression technologies; in the case of reduction or suppression, a reduction or elimination of the gene product may be determined.

The potential toxicity of the treatment may be evaluated by behavioral manifestations, and where appropriate, by analysis of biopsy specimens. Thus, behavioral activity which evidences distress, such as changes in activity level, changes in eating and drinking patterns and the like, can be monitored, as well as evidence of necrosis, edema or inflammation in biopsy specimens.

The subject compositions can be provided for use in one or more procedures. Kits will usually include the DNA either as naked DNA or complexed to lipid carriers. Additionally, lipid carriers may be provided in a separate container for complexing with the provided DNA. The DNA or the lipid carrier/DNA complexes may be present as concentrates which may be further diluted prior to use or they may be provided at the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in sterilized containers suitable for use with a nebulizer, so that the physician or veterinarian may employ the containers directly with a nebulizer, where the containers will have the desired amount and concentration of agents. Thus, the kit may have a plurality of containers containing the DNA or the DNA/lipid carrier complexes in appropriate proportional amounts, and optionally, appropriate diluent and mixing solutions.

When the containers contain the formulation for direct use, usually there will be no need for other reagents for use with the method.

Systemic administration

The recombinant coding-sequence flanked at its 5′ end by the promoter and regulatory sequences and at its 3′ end by a terminator and regulatory sequences may be introduced into a suitable cloning plasmid (e.g., pUC18, pSP72) for use in direct DNA uptake in host cells following introduction of the expression plasmid alone into the host. The nucleic acid construct also may be complexed with a carrier such as lipid carriers, particularly cationic lipid carriers. Lipid carriers can be prepared from a variety of cationic lipids, including DOTAP, DOTMA, DDAB, L-PE, and the like. Lipid carriers containing a cationic lipid, such as {N(1-(2,3-dioleyloxy) propyl}-N,N,N-triethylammonium} chloride (DOTMA) also known as "lipofectin", dimethyl dioctadecyl ammonium bromide (DDAB), 1, 2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) or lysinyl-phosphatidylethanolamine (L-PE) and a second lipid, such as distearoylphosphatidylethanolamine (DOPE) or cholesterol (Chol), are of particular interest. DOTMA synthesis is described in Felgner, *et al.*, Proc. Nat. Acad. Sciences, (USA) (1987) 84:7413-7417. DOTAP synthesis is described in Stamatatos, *et al.*, *Biochemistry* (1988) 27:3917. DOTMA:DOPE lipid carriers can be purchased from, for example, BRL. DOTAP:DOPE lipid carriers can be purchased from Boehringer Mannheim. Cholesterol and DDAB are commercially available from Sigma Corporation. DOPE is commercially available from Avanti Polar Lipids. DDAB:DOPE can be purchased from Promega. Biodegradable cationic amphiphiles also have been shown to form stable complexes with polyanionic DNA.

Cationic liposomes have been shown to be capable of mediating high level cellular expression of transgenes or mRNA by delivering the nucleic acid into a wide variety of cells in culture. The use of specific cationic lipids can confer specific advantages for *in vivo* delivery. For example, iv injection of DOTAP-containing liposomes can target transgene expression primarily to the lung. Furthermore, DOTAP, E-DC, and E-DPMC, as well as L-PE and CEBA, are fully metabolized or excreted by cells, whereas DOTMA cannot be fully metabolized by cells. Therefore, DOTAP, E-PC, E-DPMC, and L-PE, but not DOTMA, are suitable for repeated injection into mammalian hosts. Additionally, complexing the cationic lipid with a second lipid, primarily either cholesterol or DOPE can maximize transgene expression *in vivo*. For example, mixing a steroid, such as cholesterol,

instead of DOPE with DOTAP, E-DC, E-DPMC, DOTMA, or DDAB, substantially increases transgene expression *in vivo*.

Particular cells and tissues may be targeted, depending upon the route of administration and the site of administration. For example, transfection of a tissue which is closest to the site of injection in the direction of blood flow may be transfected in the absence of any specific targeting. Specific cationic lipid can target cationic lipid carriers to specific cell types *in vivo* after systemic injection. Additionally, if desired, the lipid carriers may be modified to direct the lipid carriers to particular types of cells using site-directing molecules. Thus antibodies or ligands for particular receptors may be employed, with a target cell associated with a particular surface protein. For example, with the AIDS virus, the AIDS virus is primarily directed to cells having the CD4 surface protein. By having anti-CD4 antibody bound to the surface of the lipid carrier, the lipid carrier may be directed primarily to T-helper cells. A particular ligand or antibody may be conjugated to the lipid carrier in accordance with conventional ways, either by conjugating the site-directing molecule to a lipid for incorporation into the lipid bilayer or by providing for a linking group on a lipid present in the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art. Ligand-directed DNA-polycation complexes have been shown to transfect to hepatocytes in the liver after iv injection; the ability to transfect other cell types or tissue types by this approach has not been demonstrated. Non-cationic lipid carriers, particularly pH sensitive liposomes, offer another potentially attractive approach to *in vivo* gene therapy. However, as compared to cationic liposomes, pH sensitive liposomes are less efficient in capturing DNA and delivering DNA intracellularly and may be inactivated in the presence of serum, thus limiting their iv use.

Unexpectedly, either the liposomal lipid composition or the mean diameter of the lipid carriers (when in particle form such as a liposome) injected can dramatically affect the level of transgene expression produced *in vivo*. Thus, the liposomal lipid compositions generally have a composition of 50% molar ratio of cationic lipid to non-cationic lipid, but may range from 5% to 100%. The diameter of the lipid carriers should generally be within the range of 100 nm to 10 microns. Cationic lipid carrier-DNA complexes wherein the lipid carriers range from 100 nanometers to several microns in diameter can produce significant levels of transgene expression after systemic introduction into a mammalian host.

The use of lipid carriers of greater than 500 nanometers (in other words multilamellar vesicles (MLV) or large unilamellar vesicles (LUV)) can in certain cases significantly increase the level of transgene expression achieved in a mammalian host when compared to small unilamellar vesicles (SUV). MLV and LUV are prepared by vortexing rather than sonicating after addition of the aqueous material to the dry lipid film. If desired, the resulting lipid carriers can be extruded under high pressure through sized polycarbonate membranes to achieve more uniform size distributions.

Also unexpectedly, the use of particular nucleic acid to lipid carrier ratio also is essential; the ratios used determine whether and to what level transgenes are expressed *in vivo* and needs to be optimized, depending upon various factors including the nature of the construct, the size and lipid composition of the lipid carrier and whether it is MLV or SUV, the route of administration and the host mammal. As an example, using a reporter gene CAT (chloramphenicol acetyl transferase), an approximately 1:1 (range 0.5:1 to 2:1) DNA to lipid carrier ratio (μg DNA to nmoles of the cationic lipid) produces the highest levels of gene expression in a mouse in all organs after ip administration, and an approximately 1:4 ratio, (range 2:1 to 1:7) produces the highest levels of gene expression in all organs after iv administration. In addition to achieving a high level of transgene expression in a wide variety of tissues using optimal conditions, the majority of all cells present in the lung, spleen, lymph nodes and bone marrow are transfected *in vivo*, as well as the majority of all endothelial cells present in the heart.

The DNA:lipid carrier ratio determines whether or not, and at what level, transgenes are expressed in mammalian hosts after systemic injection of the complexes. Several factors are important in order optimize the DNA:lipid carrier ratio. Thus, specific DNA:lipid carrier ratios are required for each type of cationic lipid used as well as for each different lipid carrier size used. To optimize, for each lipid carrier composition used, DNA must be mixed together with the lipid carriers in multiple different ratios, ranging from 4:1 to 1:10 (micrograms DNA to nanomoles cationic lipid), in order to determine which ratios result in aggregation of the DNA:lipid carrier complexes. Ratios which result in aggregation cannot be used *in vivo*. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confers the highest level of transgene expression *in vivo*. For example, the optimal DNA:lipid carrier ratios for SUV for DOTMA/DOPE, DDAB/DOPE, DOTAP/DOPE, DOTAP/Chol, LPE:CEBA, DDAB:Chol, L-PE:DOPE, and E-PC/chol are 1:4, 1:3, (very low activity at all ratios), 1:6, 1:1, 1:5,

2:1, and 2:1, respectively. DNA:lipid carrier complexes must be made in appropriate physiologic solutions. The DNA:lipid carrier complexes must be mixed in physiologic solutions (approximately 290 milliosmoles) which do not themselves induce aggregation of the DNA:lipid carrier complexes. The solutions include 5% dextrose in water or normal saline.

The construction of the vector itself is also critical for producing high level *in vivo* expression of the transgene after aerosol or systemic administration. Optimally, the vector either lacks an intron or contains an expanded 5" intron which does not result in aberrant splicing. In addition, a strong promoter-enhancer element, such as the AD169 strain of HEMV or the addition of a strong heterologous enhancer from for example an SV.40 or HCMVIEI gene to a weak promoter, such as that from a CFTR gene confers high level *in vivo* expression of the transgene. Using appropriately constructed vectors, high level *in vivo* expression may be obtained after systemic injection of the vector alone, or more efficiently, when complexed to a cationic lipid carrier. Furthermore, use of the CFTR promoter together with a heterobgols enhancer can be used to produce significant transgene expression in a tissue and cell-type specific fashion which approximates the endogenous pattern of FTR gene expression.

Cell surface receptors for cationic lipid carriers can be used to both regulate and confer target cell specificity on transgene expression in mammalian hosts. Cationic lipid carrier:DNA complexes are internalized by cells by a classical receptor-mediated endocytosis (see Figure 7) using cell surface receptors which contain specific binding sites for, and are able to internalize, cationic molecules. Using agents such as cytokines, growth factors, other soluble proteins and certain drugs, it is thus possible to selectively up or down regulate these cation-binding receptors. The rate of up or down regulation of these receptors by the appropriate agent will allow selection of specific cells for enhanced or reduced levels of transfection *in vivo*. Furthermore, surprisingly cell surface receptors for naked DNA can be used both to regulate and to confer target cells specificity on transgenic expression in mammalian host.

The most frequent interaction between DOTMA lipid carriers, either the uni- or multilamellar lipid carriers, complexed to plasmid DNA and the various cell types (for example, CV-1 monkey kidney cells, U937 human myelomonocytic leukemia cells, K562, MEL (murine erythroblastic leukemia cells), rat alveolar macrophages, and alveolar type II cells), is that of lipid carrier adhesion and internalization. This interaction is common to

well-defined examples of receptor-mediated endocytosis. All cells which appear to have contacted cationic lipid carrier:DNA complexes ingest the complexes after binding to the plasma membrane. All these cell types demonstrate the same classical receptor-mediated endocytic pathway of internalization.

The mammalian host may be any mammal, particularly a mammal having symptoms of a genetically-based disorder. Thus, the subject application finds use in domestic animals, feed stock, such as bovine, ovine, and porcine, as well as primates, particularly humans. The mammalian host may be pregnant, and the intended recipient of the gene-based therapy may be either the gravid female or the fetus or both. In the method of the invention, transfection *in vivo* is obtained by introducing a therapeutic transcription or expression vector into the mammalian host, either as naked DNA or complexed to lipid carriers, particularly cationic lipid carriers. The constructs may provide for integration into the host cell genome for stable maintenance of the transgene or for episomal expression of the transgene. The introduction into the mammalian host may be by any of several routes, including intravenous or intraperitoneal injection, intratracheally, intrathecally, parenterally, intraarticularly, intramuscularly, etc. Of particular interest is the introduction of a therapeutic expression vector into a circulating bodily fluid. Thus, iv administration and intrathecal administration are of particular interest since the vector may be widely disseminated following such a route of administration. Any physiologically acceptable medium may be employed for administering the DNA or lipid carriers, such as deionized water, saline, phosphate-buffered saline, 5% dextrose in water, and the like, depending upon the route of administration Other components may be included in the formulation such as buffers, stabilizers, biocides, etc. These components have found extensive exemplification in the literature and need not be described in particular here.

The amount of lipid carriers used will be sufficient to provide for adequate dissemination to a variety of tissues after entry of the DNA or complexes into the bloodstream and to provide for a therapeutic level of expression in transfected tissues. A therapeutic level of expression is a sufficient amount of expression to treat or palliate a disease of the host mammal. In addition, the dose of the plasmid DNA expression vector used must be sufficient to produce significant levels of transgene expression in multiple tissues *in vivo* for example, $\geq$ 1mg of an expression plasmid alone is injected into a mouse to achieve high level expression of the CAT gene in multiple tissues. Other DNA sequences, such as adenovirus VA genes can be included in the administration medium and

be co-transfected with the gene of interest. The presence of genes coding for the adenovirus VA gene product may significantly enhance the translation of mRNA transcribed from the plasmid.

The level and tissues of expression of the recombinant gene may be determined at the mRNA level and/or at the level of polypeptide or protein. Gene product may be quantitated by measuring its biological activity in tissues. For example, enzymatic activity can be measured by biological assay or by identifying the gene product in transfected cells by immunostaining techniques such as probing with an antibody which specifically recognizes the gene product or a reporter gene product present in the expression cassette. Alternatively, potential therapeutic effects of the gene product can measured, for example where the DNA sequence of interest encodes GM-CSF, by determining the effects of gene expression on survival of lethally irradiated animals in which the GM-CSF transgene is expressed. Production of significant amounts of a transgene product will substantially prolong the survival of these mice.

Where expression of the polypeptide/protein or even the mRNA itself confers a changed biochemical phenotype upon the host, the presence of a new phenotype or absence of an old phenotype may be evaluated; for example, as a result of transfection of the host cells, there may be enhanced production of pre-existing desirable products formerly produced in insufficient quantities or there may be reduction or even suppression of an undesirable gene product using antisense, ribozyme or co-suppression technologies; in the case of suppression, a reduction of the gene product may be determined. Typically, the therapeutic cassette is not integrated into the host cell genome. If necessary, the treatment can be repeated on an ad hoc basis depending upon the results achieved. If the treatment is repeated, the mammalian host can be monitored to ensure that there is no adverse immune response to the treatment.

The subject compositions can be provided for use in one or more procedures. Kits will usually include the DNA either as naked DNA or complexed to lipid carriers. Additionally, lipid carriers may be provided in a separate container for complexing with the provided DNA. The DNA either for direct injection or for complexing with lipid carriers, or the lipid carrier/DNA complexes may be present as concentrates which may be further diluted prior to use or they may be provided at the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in syringes, contained in sterilized containers, so that the physicians or veterinarian may employ the

syringes directly, where the syringes will have the desired amount and concentration of agents. Thus, the kit may have a plurality of syringes containing the DNA or the DNA/lipid carrier complexes in appropriate proportional amounts. When the syringes contain the formulation for direct use, usually there will be no need for other reagents for use with the method.

The invention finds use in *in vivo* treatment and/or palliation of a number of diseases. *In vivo* replacement of a gene can be accomplished by techniques such as homologous recombination or initial knockout of the aberrant gene and subsequent replacement with the desired transgene.

Uses

Uses of the subject invention include but are not limited to the following. The present invention is particularly useful for the delivery of substances into the lung and appropriate extrapulmonary tissues for the prevention and/or treatment of the multi-organ system manifestations of CF. Specifically, it is useful for the prevention, treatment, and cure of the disease manifestations of CF in tissues, including the lung, liver, pancreas, and colon.

For the treatment of cystic fibrosis a functional CFTR gene, or a nucleic acid sequence encoding a molecule having wild-type CFTR activity is administered. The gene can be administered prophylactically, as well as in response to clinical manifestations of the disease, for both the prevention and/or treatment of this disorder. The invention also finds use for the delivery of substances into the systematic circulation via the lung. The amount of CFTR produced can be controlled by modifying the dose administered, the frequency and duration of dosing, the strength of the promoter and enhancer elements used to direct transcription of the transgenes and the efficiency and target specificity of the lipid carrier user.

The instant methods also find use in antisense therapy, for the delivery of oligonucleotides able to hybridize to specific complementary sequences of a defective or mutant CFTR gene, thereby inhibiting the transcription and/or translation of these sequences. Thus, DNA or RNA coding for proteins necessary for the progress of a particular disease, can be targeted, thereby disrupting the disease process. For a review of antisense therapy and oligonucleotides useful in the same, see, Uhlmann, E. and Peyman, A., *Chem. Rev.* (1990) 90:543-584.

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the present invention.

EXAMPLES

The practice of the present invention employs unless otherwise indicated, conventional techniques of cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Vols. 1-3; DNA Cloning (1985) Vols. I and II, D.N. Glover (ed.); Nucleic Acid Hybridization (1984), B.D. Hames, *et al.*, (eds.); Perbal, B., A Practical Guide to Molecular Cloning (1984); Methods in Enzymology (the series), Academic Press, Inc.; Vectors: A Survey of Molecular Cloning Vectors and Their Uses (1987), R.L. Rodriguez, *et al.*, (eds.), Butterworths; and Miller, J.H., *et al.*, Experiments in Molecular Genetics (1972) Cold Spring Harbor Laboratory.

We have not modified the INTOX chamber. Up to 48 mice can be exposed simultaneously to an aerosol dose. Approximately 0.02% of the total volume of DNA:liposome complex solution placed in the nebulizer is actually deposited in the lungs of each individual mouse.

Example 1

Preparation of Plasmids for *in vivo* Gene Therapy

Details regarding the plasmids that have been used for transfection of mammalian cells are as follows.

pRSVCAT: construction of this plasmid is described in Gorman *et al.*, *Proc. Nat. Acad. Sciences (USA)* (1982), 79:6777-6781. In the pRSVCAT plasmid, the 3'-RSVLTR is juxtaposed as a promoter upstream from CAT encoding sequences. The distance between the LTR transcriptional start site and the CAT initiation codon (the first AUG downstream from the start site) is about 70 bp.

p5'PRL3-CAT: construction of this plasmid is described in Sakai *et al.*, *Genes and Development* (1988) 2:1144-1154.

pSIS-CAT: construction of this plasmid is described in Huang and Gorman, *Nucleic Acids Research* (1990) 18:937-948.

pZN20: construction of this plasmid is illustrated in Figure 3. The plasmid was prepared as follows. pCATwt760 (Stinski and Roehr (1985) *J. Virol.* 55:431-441) was treated with *Hin*dIII and the fragment containing the HCMV base IE 1 enhancer and promoter element purified. The isolated fragment was then cloned into the *Hin*dIII site of pSP72 (Promega) creating pZN9. Clones were screened in which the enhancer and promoter element is as shown in Figure 3. Following partial *Hin*dIII digestion of pZN9, the blunt ends were filled in with DNA polymerase I Klenow fragment. The resulting clone pZN12 has lost the *Hin*dIII site 5′ to the enhancer and promoter element. pZN12 was then treated with *Nco*1 and *Hin*dIII and the large *Nco*1-*Hin*dIII fragment purified and ligated to a purified small *Nco*1-*Hin*dIII fragment from pBC12/CMV/IL-2 (Cullen, *Cell* (1986) 46:973-982. pBC12/CMV/IL-2 contains the HCMV promoter from the AD169 strain. The resulting clone was pZN13. pZN13 was partially digested with *Bam*H1, filled in with DNA polymerase I Klenow fragment and the resulting clones screened for the clone which has lost the *Bam*HI site at the 5′ end of the enhancer and promoter element. The resulting clone was called pZN17. pZN17 was treated with *Hin*dIII and *Bam*HI and the resulting *Hin*dIII-*Bam*HI large fragment was purified and ligated to a purified small *Hin*dIII-*Bam*HI fragment obtained from pSV2-CAT (Gorman *et al.* (1982), *Molecular Cell Biology*, 2:1044-1051). The resulting clone was pZN20. The full restriction map of HCMV (Towne) is shown in Figure 19A. HCMV (AD169) is shown in Figure 19C. A comparison of the two promoters is shown in Figure 6B. Significantly more expression is obtained when a promoter from the AD169 strain is used as compared to one from the Towne strain. pZN20 contains a composite promoter which has the Towne sequence 5' of the NcoI site and the AD169 sequence 3' of the NcoI site. The NcoI site is indicated by the asterisk in Figure 19B. pZN20 has this composite HCMV promoter followed by the CAT gene, SV40 t-intron and SV40 polyA addition site.

pZN27: Construction of this plasmid is illustrated in Figure 7. pZN27 contains the composite HCMV promoter followed in order by the SV40 t-intron, the CAT coding sequence and the SV40 polyA addition site.

pZN46: Construction of this plasmid is shown in Figure 27A and Figure 27B. pZN46 contains the composite HCMV promoter, followed by the human IL-2 gene, rat preproinsulin 2 intron and polyA addition site from the rat preproinsulin 2 gene. These last three components were derived from pBC12/CMV/IL-2 plasmid of Cullen (Cell 46:973-982

(1986). The rat preproinsulin2 intron was modified by deleting an internal 162 base pair NdeI fragment.

pZN32: Construction of this plasmid is shown in Figure 10. pZN32 contains the composite HCMV promoter followed in order by the modified rat preproinsulin2 intron described for pZN46, CFTR cDNA, and rat preproinsulin2 gene polyA addition site as described for pZN46. CFTR cDNA was obtained from pBQ4.7 from F. Collins (Univ. of Michigan).

pZN51: Construction of this plasmid is shown in Figure 11. pZN51 contains the composite HCMV promoter followed by the CAT coding sequence and the SV40 polyA site.

pZN60, pZN61, pZN62, pZN63: Construction of these plasmids is shown in Figure 19. pZN60 contains the HCMV composite promoter followed by the modified rat preproinsulin 2 intron, the CAT coding sequence, and the SV40 polyA addition site. pZN61 is identical to pZN60 but contains an additional 166 base pairs 5' to the intron. This additional DNA is the 166 BP immediately 5' of the intron in the pBC12/CMV/IL-2 plasmid and may contain rat preproinsulin 2 gene coding sequence. pZN62 is similar to pZN60 except that the intron is 3' of the CAT coding sequence rather than 5' as in pZN60. pZN63 is identical to pZN62 except for the additional 166 base pairs 5' to the intron. This is the same additional sequence described for pZN61.

Example 2

Expression of chloramphenicol acetyltransferase (CAT) gene, in rodent lungs following aerosolized delivery of lipid carrier-nucleic acid complexes.

The lipid carriers used were plasmid pRSV-CAT, as described by Gorman, *et al.*, *Proc. Natl. Acad. Sci. USA* (1982) 79:6777-6781; and Juang, and Gorman, *Mol. Cell. Biol.* (1990) 10:1805-1810; a plasmid containing the CAT gene driven by the RSV long terminal repeat; and plasmid pRSV-β-gal, as described by Hazinski *et al.*, *Am. J. Respir. Cell Mol. Biol.* (1991) 4:206-209.

The pRSV-CAT plasmid was complexed to lipid carriers and administered to 25 gram female BALB/c mice as follows. Two mg of pRSV-CAT was mixed with 4 μmoles of DOTMA (GIBCO BRL, Grand Island, NY)/cholesterol (2:1) small unilamellar liposomes in phosphate buffered saline and then nebulized in an Acorn I nebulizer (Marquest Medical Products, Inc., Inglewood, CO) to groups of rats or mice in an Intox nose-only exposure

chamber (Intox Products, Albuquerque, NM). The same procedure was followed with 0.5 mg pRSV-CAT mixed with 1.0 μmol DOTMA-cholesterol (2:1), as well as 2.0 mg pRSV-CAT alone. Two to five days later, animals were sacrificed and lungs collected. Lungs were also collected from untreated controls. The lungs were homogenized and cells disrupted with three freeze-thaw cycles. CAT activity in aliquots from the lung extracts was measured using a standard assay as described by Wolff, *et al.*, *Science* (1990) 247:1465-1468.

Results

As can be seen in Figure 20, animals administered 2.0 mg RSV-CAT with 4.0 μmol DOTMA/cholesterol (2:1) expressed the CAT protein while the control animals, as well as animals receiving RSV-CAT DNA abre and animals receiving a lower dose of RSV-CAT-DOTMA:chol complexes did not. A similar procedure was followed with respect to pRSV-β-gal, with the exception that 50 mg of pRSV-β-gal was mixed with 50 μmoles of DOTMA/cholesterol (2:1). The presence of β-gal activity was determined using a standard histochemical staining procedure. β-gal activity was present in the airway epithelial cells of exposed rats.

Also tested was a plasmid containing the CAT gene driven by the CMV promoter. This plasmid was made as described in Huang, M.T.F. and Gorman, C.M. *Nuc. Acids Res.* (1990) 18:937-947, with the exception that a CMV promoter and a hybrid intron sequence were used rather than the SV40 promoter in the plasmid pML.I.CAT, described therein. Briefly, the CAT lipid carrier was constructed by first making a pML-based plasmid containing the CMV promoter immediately followed by a portion of the 5'-untranslated leader from the adenovirus-major late (AML) region. This region contained all but the first 13 nucleotides of the first exon of the tripartite leader plus a portion of an intervening sequence (IVS) from the AML region. A synthetic oligonucleotide was inserted which merged with the adenovirus intron to provide a functional splice acceptor sequence derived from an IgG variable region. Bothwell, *et al.*, *Cell* (1981) 24:625-637. This plasmid was then cut at two restriction sites bordering the intron (ClaI and PstI) to remove a 292 bp fragment. A matching synthetic oligonucleotide linker was inserted. The plasmid was termed pCIS-CAT.

To test for expression of the CAT gene using pCIS-CAT, 12 mg pCIS-CAT was mixed with 24 μmoles of DOTMA/DOPE (1:1). Female ICR mice were placed in three different aerosol receiving chambers. All mice received the same amount of the CAT

expression plasmid complexed to liposomes, as described above. Animals 1-3 were exposed to the aerosol in an Intox designed aerosol chamber. Animals 4-7 were exposed to the aerosol in a modified rat cage containing dividers for individual mice. Animals 8-10 were placed in a smaller, similarly modified mouse cage after being put in the restrainers used in the Intox chamber. 48 hours following aerosolization, the animals were sacrificed and whole lungs assayed for CAT expression using the chromatographic CAT assay. As can be seen in Figure 21, a single aerosol dose of a CAT gene-expression plasmid complexed to cationic liposomes can produce high-level transgene expression in the lungs of mice. Significant levels of transgene expression are present in the lungs of all 7 mice (numbers 1-3 and 8-10) which were exposed to the aerosol mist in Intox nose-only exposure tubes which were constructed to maximize the amount of aerosol that the mice inhaled. The amount of variation seen here is comparable to that seen in other aerosol experiments and may have several explanations, including variations in exposure to the aerosol mist, individual variations in efficiency of nasal filtration, etc.

Example 2

Preparation of Lipid carriers and DNA Complexing with Lipid carriers

Lipid carriers containing a cationic lipid, such as {N(1-2-3-dioleyloxy) propyl}-N,N,N-triethylammonium} (DOTMA), dimethyl dioctadecyl ammonium bromide (DDAB), or 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) or lysinyl-phosphatidylethanolamine and a second lipid, such as dioylphosphatidylethanolamine (DOPE) or cholesterol, were prepared as follows.

Preparation of lipid carriers:

Lipids, e.g. DDAB, L-lysinyl-phosphatidylethanolamine (L-PE), E-PC, E-DMPC, cholesterol-ester-β-alanine (CEBA), DOTAP, and cholesterol (Chol) were dissolved in chloroform. Proper amounts of each lipid (determined by the desired molar ratio of each lipid in the final lipid carrier formulation usually 1 to 1 moles cationic lipid to moles non-cationic lipid but ranging from 5 to 1 to 1 to 5) were mixed together and evaporated to dryness on a rotary evaporator. The lipid film was then resuspended by vortexing after the addition of 5% dextrose in water or lipid carrier buffer (25 mM Tris-HCl pH7.4, 100 μM $ZnCl_2$ isotonic solution) to make a final lipid concentration of 20mM of multi-lamellar

vesicles (MLV). For the preparation of small unilamellav vesicles (SUV), the mixture was then sonicated in a bath sonicator for 15 min, and the lipid carriers were stored under argon at 4°C until use.

Plasmid Preparation:

The *E. coli* strain which carries the plasmid was grown in TB at 37°C. The method of plasmid purification is a modification of the protocol of "lysis by alkali" and "purification of plasmid DNA by precipitation with polyethylene glycol" described by Sambrook, *et al.* (*Molecular Cloning,* 2nd edition, 1989, Cold Spring Harbor Laboratory Press). The modification is that the precipitation of DNA by PEG is omitted. The final DNA preparation is dissolved in 10mM Tris-HCl pH8.0.

Preparation of lipid carrier-plasmid complexes:

Plasmids were diluted separately in 5% dextrose in water solution to the desired concentration (usually 1 μg/μl). The lipid carriers were also diluted in 5% dextrose in water to the same volume as the plasmid.

The amounts of lipid carriers used were determined based on the ratio of moles of liposomal lipid to μg of plasmid added, e.g. for lipid carrier:plasmid=1:1, one nanomole of cationic lipid is mixed with 1 μg of plasmid DNA. Plasmid and lipid carriers were then mixed together to form DNA:lipid carrier complexes.

Dose injected. At least 50 μg, and routinely 100 μg of plasmid DNA complexed to cationic lipid carriers is injected per mouse. For injection of plasmid alone, at least 500 μg and routinely 2 mg of plasmid DNA is injected by tail vein per mouse.

Example 3

Demonstration by Immunohistochemistry of CAT Gene Expression in the Lung After Intravenous (iv) injection of pZN27-DDAB: Cholesterol Lipid carrier Complexes

Lipid carrier: DDAB:Chol=1:1, stock 20 mM in lipid carrier buffer.

Plasmid: pZN27.

DNA:Lipid carrier Ratio: lipid carrier:plasmid=5 nanomoles cationic

lipid:1 μg DNA

DNA dose: 100 μg plasmid DNA in 200 μl 5% dextrose in water was injected iv by tail vein per mouse.

Mice: ICR, female, 25 grams.

Immunohistochemical staining to detect CAT protein in lung sections of mice treated *in vivo*.

Procedure: Forty eight hours after injection of the pZN27-DDAB:Chol complexes, the lungs are removed, perfused with 33% O.C.T., embedded in O.C.T. and snap frozen. Frozen tissues are sectioned at 6 microns, collected onto glass slides, fixed for 10 minutes in 4°C acetone and then placed in 0.2% Triton X-100 to permeabilize membranes. Sections are then incubated for 12-48 hours with the monoclonal anti-CAT antibody (available from Dr. Parker Antin, Univ. of Arizona) or isotype negative control antibody at the appropriate dilution. After washing, 1) a biotinylated antibody directed against the primary antibody (Zymed, S. San Francisco) is added for a minimum of 60 minutes, 2) followed by application of the streptavidin-alkaline phosphatase complex (Zymed) for 60 minutes and 3) application of the substrate-chromogen appropriate for the enzyme label per manufacturers instructions. Slides are then coverslipped in water-soluble mounting media for examination.

Results: The results are shown in Fig. 2A, 2B and demonstrate diffuse staining of the lung. The stain localizes to the alveolar walls, indication that greater than 70% of pulmonary vascular endothelial cells, as well as alveolar lining cells, including type I and type II cells and alveolar macrophages are transfected by a single iv injection of DNA lipid carrier complexes. In addition, significant numbers of bronchiolar airway lining cells stain positively for CAT protein, and are therefore transfected *in vivo* by iv injection of lipid carrier:DNA complexes. Thus, the great majority of all cells in the lung transfected by a iv injection of pZN27-DDAB:CHOL complexes.

Example 4

Expression of pZN20

Following Intraperitoneal Administration

Effect of the Amount of pZN20-cationic lipid carrier complexes injected ip on the level of CAT gene expression *in vivo*.

Female ICR mice (Simonson Labs, Gilroy, CA) were injected ip with 1 ml of 5% dextrose in water containing 0.01, 0.1 or 1 mg of pZN20 expression plasmid complexed to 0.01, 0.1 or 1 μmoles, respectively of DDAB:DOPE lipid carriers. Mice were sacrificed 48 hours later, the organs removed, and tissues were homogenized in 0.25M Tris-HCL buffer pH 7.8, using a hand-held homogenizer. Cytoplasmic extracts were made, normalized by protein content and level of CAT protein was then measured. The experiments comprise three animals per group and the results show the mean dpm $\pm$ SEM of acetylated chloramphenicol.

Methods: Lipid carriers containing DDAB were prepared in 1:1 molar ratio with DOPE, as follows: 10 μmoles of DOPE dissolved in chloroform and 10 μmoles of the cationic lipid, dissolved in ethanol were evaporated to dryness on a rotary evaporator. One ml sterile of water was added, and the mixture was sonicated in a bath sonicator (Laboratory Supply, Hicksville, NY) for 20 min. Lipid carriers had mean diameters of approximately 100 $\pm$25 nm. For CAT assay, cell extracts were made, and their protein content determined by the Coomasie blue assay (BioRad, Richmond, CA). One hundred μg of protein from the lung, spleen, liver, and heart extracts, and 50 μg of lymph node extract were reacted with ^{14}C labeled chloramphenicol and chromatographed as previously described (Gorman, *supra*). To calculate dpm, both the acetylated and unacetylated species were cut from TLC plates and radioactivity counted in a scintillation counter. The ratio between acetylated and unacetylated counts was used to calculate the mean dpm. The mean dpm from tissues of untreated control animals were subtracted from each treated animal for each tissue.

Results: To assess potential dose-response relationships *in vivo*, animals were injected animals in groups of three with 0.01 mg, 0.1 mg, or 1 mg of pZN20 plasmid complexed to 0.01 μmole, 0.1 μmole, or 1 μmole respectively of DDAB:DOPE lipid carriers. Both the 0.1 mg and 1 mg DNA doses produced highly significant levels of CAT protein ($p < 0.005$) in all the organs assayed. Maximal levels of CAT gene expression in

each organ were produced by the 1 mg DNA dose: increasing the DNA-lipid carrier dose 10 fold resulted in an approximately 2 fold increase in lymph node CAT levels and a 3 fold increase in the spleen. Intraperitoneal injection of 1 mg of the pZN20 plasmid alone did not produce detectable CAT protein above background levels.

Example 5

Demonstration of CAT gene expression in the spleen after intravenous (iv) injection of p5'PRL3-CAT:L-PE:CEBA complexes.

Lipid carrier: L-PE:CEBA=1:1, stock 20 mM in lipid carrier buffer.

Plasmid: p5'PRL3-CAT.

DNA:Lipid carrier Ration: lipid carrier:plasmid=1 nanomole cationic lipid: 1 μg plasmid DNA.

DNA dose: 200 μg plasmid DNA in 200 μl 5% dextrose in water was injected by tail vein per mouse.

Mice: BalB/c, female, 25 grams.

Tissue extraction procedure: Forty eight hours after tail vein injection, mice were sacrificed, whole spleen was homogenized in 1ml of 0.25M Tris-HCl pH 7.8, 5mM EDTA, 80 μg/ml PMSF and the resulting extract was centrifuged and then the supernatant was subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min.

CAT assay procedure: 100 μl of extract+10 μl of 20 mM acetyl CoA+4 μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole, Amersham) were incubated together at 37°C for 6 hr. At 3 hours, an additional 10 μl of acetyl CoA was added.

Results: The results are lane 2 (lipid carriers only) and lane 5 (lipid carrier-DNA complex), and indicate that a significant level of CAT activity is present in the spleen extract of the treated animal, but not in the extract of control spleen, taken from an animal injected with lipid carrier alone.

Demonstration of CAT gene expression in the lung after intravenous (iv) injection of pRSV-CAT:L-PE:CEBA complexes.

Lipid carrier: L-PE:CEBA=1:1, stock 20 mM in lipid carrier buffer.

Plasmid: pRSV-CAT.

DNA:Lipid carrier Ratio: lipid carrier:plasmid=1 nanomole cationic lipid: 1 μg plasmid DNA.

DNA dose: 100 μg plasmid DNA in 200 μl 5% dextrose in water was injected by tail vein per mouse.

Mice: BalB/c, female, 25 grams.

Tissue extraction procedure: Forty eight hours after tail vein injection, the animals were sacrificed, whole lung was homogenized in 1ml of 0.25M Tris-HCl pH 7.8, 5mM EDTA, 80 μg/ml PMSF and the resulting extract was centrifuged and then the supernatant was subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min..

CAT assay procedure: 100 μl of extract+10 μl of 20 mM acetyl CoA+4 μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole, Amersham) were incubated together at 37°C for 6 hr. At 3 hours, an additional 10 μl of acetyl CoA was added.

Results:

The results are shown in Figure 9, and indicate that a significant level of CAT activity (indicative of expression of the transgene) was present in the lung of the animal injected with lipid carrier:DNA complexes (lane 5), but not present in the lungs from control animals (lanes 1-4).

Demonstration of CAT gene expression in multiple tissues after intravenous (iv) injection of pZN20:DDAB:DOPE complexes.

Lipid carrier:

DDAB:DOPE=1:1, stock 10mM in 5% dextrose.

Plasmid: pZN20.

DNA:Lipid carrier Ratio:

lipid carrier:plasmid=(A) 3 nanomole cationic lipid:1 μg plasmid DNA (SUV);(B) 6 nanomole cationic lipid:1 μg plasmid DNA (MLV).

DNA dose:

100 μg plasmid DNA in 200 μl 5% dextrose in water was injected by tail vein per mouse. Three mice each received this dose of MLV:pZN20 and 3 mice each this dose of SUV:pZN20.

Tissue extraction procedure:

Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the resulting extract was centrifuged and then the supernatant was subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min..

CAT assay procedure:

The protein concentration of each tissue extract was quantitated using a Coomasie blue-based protein assay (Bio-Rad, Richmond, CA), and the same amount of total protein from each tissue extract was added in the CAT assay, together with 10 μl of 20 mM acetyl CoA+12μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole, Amersham), at 37°C for 13 hrs.

Results:

The results are shown in Figure 5, and demonstrate that iv injection of pZN20:DDAB:DOPE complexes significant levels of CAT gene expression in each of 6 different tissues including lung, heart, liver, spleen, kidney and lymph nodes. Furthermore, MLV lipid carriers mediate equal or higher levels of *in vivo* transgene expression than do SUV lipid carriers composed of the same lipids.

Demonstration of CAT gene expression *in vivo* after intravenous (iv) injection of pZN20 alone.

Plasmid: pZN20.

DNA:Lipid carrier Ratio: Plasmid DNA alone, without lipid carriers, was injected.

DNA dose: 300 μg plasmid DNA in 200 μl 5% dextrose in water was injected by tail vein per mouse.

Mice: ICR, female, 25 grams.

Tissue extraction procedure: each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the resulting extract was centrifuged and then subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min.

CAT assay procedure: the protein concentration of each tissue extract was quantitated using a ninhydrin-based protein assay (Bio-Rad, Richmond, CA), and same amount of total protein from each tissue extract was added in the CAT assay, together with 10 μl of 20 mM acetyl CoA+12 μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole, Amersham) at 37°C for 13 hrs.

Example 6

Injection of DOTMA:DOPE+pSIS-CAT Plasmid Clearly Did Not Produce Detectable CAT Gene Expression *in vivo*)

Lipid carrier: DOTMA:DOPE=1:1, in 5% dextrose in water

Plasmid: pSIS-CAT (Huang, M. T. F. and C. M. Gorman, 1990, *Nucleic Acids Research* 18:937-947).

Ratio: Cationic lipid:plasmid=4 nmoles: 1 μg, dose: 100 μg DNA in 200 μl 5% dextrose in water.

Mouse: ICR, female, 25 grams.

Injection: tail vein.

Tissue collection and processing:

Mice were sacrificed at day 2 and day 6, and lung, spleen, liver, and heart were collected. The whole organs were homogenized in 0.5 ml, except livers which were homogenized in 2.0 ml, of 0.25M Tris-HCl pH 7.8, 5 mM EDTA, 2 μg/ml aprotintin, 1 μg/ml E-64, and 0.5 μg/ml leupeptin (all protease inhibitors were purchased from Boehringer Mannheim). Extracts were subjected to three cycles of freeze-thaw, then heated to 65°C for 10 min.

CAT assay: 100 μl of extracts for each assay with 0.3 μCi of ^{14}C-chloramphenicol and 10 μl of 20 mM acetyl CoA at 37°C for either 5 hrs or 24.5 hrs, and the materials were then extracted using ethyl acetate and analyzed on TLC plates.

Result: There were no acetylated chloramphenicol species presented as determined by comparing the extracts from treated animals with that from control animals.

Thus, under similar experimental conditions that produce high level expression of pZN27, the use of the pSIS-CAT expression vector does not result in any detectable expression of the linked-CAT gene in any of the tissues assayed *in vivo*. The lack of expression of pSIS-CAT *in vivo* may be due either to a different promoter-enhancer element (SV40) or to a different intron sequence when compared to the pZN27 vector, which yields high level *in vivo* expression.

The results are shown in Figure 6.

Example 7

Interaction Of DNA: Lipid carrier Complexes With Cell Surface Receptors

Cells and cell culture: CV-1 (African green monkey kidney), U937 (human myelocytic leukemia), murine erythroleukemia (MEL) cells, and K562 cells (human erythroleukemia cells were obtained from the American Type Culture Collection (Rockville, MD). CV-1 and MEL cells were maintained in Dulbecco minimum essential medium (DME)- H-21 with 5% fetal bovine serum (FBS) at 37°C and 7% CO_2. Rat alveolar type II cells and rat alveolar macrophages were isolated and purified as previously described. (Debs *et al. Amer. Rev. Respiratory Disease* (1987) 135:731-737; Dobbs, L. *Amer. Rev. Respiratory Disease* (1986) 134:141-145) Type II cells were maintained in DME-H-16 with 5% FBS at 37°C and 7% CO_2. Twenty nanomoles of DOTMA:DOPE lipid carriers complexed to 20 μg of pRSV-CAT plasmid DNA were added to 2 million cells growing in 60 mm Falcon plastic dishes (either SUV or MLV), and fixed for EM at time points from 15 minutes to 2 hours thereafter.

Fixation and Processing for Electron Microscopy

DOTMA lipid carriers and cells in tissue culture or freshly isolated from blood or pulmonary alveoli were fixed in 1.5% glutaraldehyde in 0.1 molar sodium cacodylate buffer containing 1% sucrose, pH 7.4, at room temperature for 1 hr. Following tannic acid and uranyl acetate enhancement, tissue was dehydrated in a graded series of alcohols and embedded in epoxy 812 resin (Ernest F. Fullam, Inc., Latham, NY) sectioned on an MT 2

microtome using diamond knives and examined with a Jeol 100CX transmission electronmicroscope operating at 80 kv. The results are shown in Figure 4.

Results

The most frequent interaction between DOTMA lipid carriers, either uni- or multilammelar lipid carriers, complexed to plasmid DNA and the various cell types (CV-1 monkey kidney cells, U937 human myelomonocytic leukemia cells, K562, MEL erythroblastic leukemia cells, rat alveolar macrophages, and alveolar type II cells), is that of lipid carrier adhesion and internalization in a typical coated vesicle pathway (Fig. 4 a-f). This interaction is common to well defined examples of receptor-mediated endocytosis. All cells which appear to have contacted cationic lipid carrier:DNA complexes ingest the complexes after binding to the plasma membrane. All these cell types demonstrate the same classical receptor-mediated endocytic pathway of internalization. Human cells are more efficiently transfected than are other cells, such as rodent cells.

Example 8

Demonstration of High Level CAT Gene Expression in Multiple Tissues After Intravenous (iv) Injection of pZN27 Alone, or pZN27:DDAB:cholesterol SUV Complexes.

Lipid carrier:

DDAB:Chol=1:1, stock 10mM in 5% dextrose. After addition of 5% dextrose to the dried lipid film, the SUV were prepared by sonication in a bath sonicator for 20 minutes.

Plasmid: pZN27.

DNA:Lipid carrier Ratio:

Cationic lipid:plasmid DNA = 5 nanomoles:1 μg DNA.

DNA dose:

pZN27 alone: Individual mice received 500 μg, 1 mg, 2 mg, or 500 μg, followed 4 hours later by a second 500 μg dose, respectively of pZN27 in 200 μl 5% dextrose in water by tail vein injection.

pZN27 complexed to lipid carriers: 100 μg plasmid DNA complexed to 500 nanomoles to DDAB:Chol SUV lipid carriers in 200 μl 5% dextrose in water was injected by tail vein per mouse.

Mice: ICR, female, 25 grams.

Tissue extraction procedure:

Each organ was homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the resulting extract was centrifuged and the supernatant was then subjected to 3 cycles of freeze-thaw and then heated to 65°C for 20 min.

CAT assay procedure:

The protein concentration of each tissue extract was quantitated using a Coomasie blue-based protein assay (Bio-Rad, Richmond, CA), and same amount of total protein from each tissue extract was added in the CAT assay, together with 10 μl of 20 mM acetyl CoA+12 μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole, Amersham)), at 37°C for 13 hrs.

Results:

The results are shown in Figure 8. Significant levels of CAT gene expression were seen in each of the 6 different tissues (lung, heart, liver, kidney, spleen and lymph nodes) assayed after injection of either pZN27 alone, or pZN27 complexed to DDAB:cholesterol lipid carriers. Expression of a transgene in multiple tissues *in vivo* after systemic injection of a naked expression plasmid has not been demonstrated previously.

Example 9

High level airway expression of the human CFTR gene in mouse lungs after aerosol administration of DDAB:cholesterol liposome-pZN32 complexes

Animals.

Two months old, female, ICR mice obtained from Simonsen, Gilroy, CA, were used.

Preparation of plasmid DNA.

The plasmid used, pZN32, contains the human CFTR gene coding region fused to the human cytomegalovirus immediate early promoter-enhancer element shown in Figures 3-5 attached hereto. A full restriction map of the immediate early enhancer and promoter region of HCMV (Towne) and HCMV (AD169) is provided in Figs. 19A and 19C. The two sequences are compared in Fig. 19B. pZN32 was purified using alkaline lysis and ammonium acetate precipitation, and the nucleic acid concentration measured by UV absorption at 260 nm.

Preparation of cationic lipid carriers.

Lipid carriers were prepared as small unilamellar vesicles (approximately 100 nm in diameter) containing the cationic lipid DDAB (dimethyl dioctadecyl ammonium bromide) as DDAB: cholesterol in a 1:1 molar ratio. DDAB was purchased from Sigma, St. Louis, MO, and cholesterol was purchased from CalBioChem, San Diego, CA. Stock solutions of the lipids were dissolved in chloroform. Lipids were mixed in a round-bottomed flask and evaporated to dryness on a rotary evaporator under reduced pressure. Double distilled water was added to produce final lipid concentrations of 10 mM each, and the resulting mix was sonicated for approximately 20 minutes in a bath sonicator (Laboratory Supplies, Hicksville, NY).

Aerosol delivery of plasmid/lipid carrier complexes to mice.

Twelve mg of pZN32 complexed to 24 μmols of DDAB:cholesterol (1:1) liposomes was aerosolized over two different aerosol periods on the same day. To prevent aggregation and precipitation of the oppositely charged components, the liposomes and DNA were diluted separately in sterile water prior to mixing. Six mg of plasmid DNA and 12 μmols of DDAB:cholesterol (1:1) liposomes were each diluted to 8 ml with water and mixed. Four ml of the DNA-liposome mixture was then placed into two Acorn I nebulizers (Marquest, Englewood, CO), and the animals placed in an Intox small animal exposure chamber (Albuquerque, NM). An air flow rate of 4 L min^{-1} was used to generate the aerosol. Ninety minutes were required to aerosolize this volume (4 ml) of DNA-liposome mixture. The animals were removed from the chamber for 1-2 hours and then the above procedure was repeated with a second 4 ml dose.

Immunohistochemical staining for the human CFTR protein in mouse lungs.

At selected time points following aerosolization, mice were sacrificed and their lungs immediately removed. The lungs were slowly inflated with phosphate buffered saline (PBS) containing 3.3% by volume OCT (Miles, Inc.), then placed in a tissue cassette filled with

OCT, and frozen in 2-methylbutane chilled in a dry ice/ethanol bath. Cryosections were cut at 5 μm and collected onto sialinized slides. CFTR protein was detected after fixation of cryosections for 10 minutes in either 4% acetone or 2% paraformaldehyde in PBS containing 0.1% Tween 20 (PBST). All subsequent dilutions and washes were done in PBST. Following fixation, sections were washed three times (5 minutes each) with PBST then covered with 10% normal rabbit serum for 10 minutes at 20°C. Immunolocalization of CFTR was then performed using an affinity purified rabbit polyclonal anti-CFTR antibody, α-1468, provided by Dr. Jonathan Cohn, Duke University. The serum was replaced with α-1468, diluted (1:1000). The antibody-covered section was gently overlaid with a siliconized coverslip and incubated in a humid chamber at 4°C for 24 hours. Slides were then warmed to 20°C and washed three times. The presence of bound rabbit antibody against CFTR was detected by covering sections with biotinylated, affinity-purified, goat anti-rabbit antibody (Lipid carrier Laboratories), diluted 1:300 for 1 hour, followed by washing (3 x 10 minutes) and replacement with streptavidin labelled with alkaline phosphatase (Zymed, South San Francisco) for 20 minutes. Immobilized alkaline phosphatase was detected using AP-red (Zymed) as the chromogen; endogenous alkaline phosphatase was inhibited with levamisole (Zymed). Other controls, run concurrently, included the use of normal rabbit serum in place of primary antibody and the use of lung tissue from untreated mice. Photo-microscopy was performed using Kodak Ektachrome 64T film at X50 and X250.

Results

Photomicrographs of frozen sections (viewed at different magnifications) of mouse lung 48 hours following aerosol exposure to pZN32-DDAB:cholesterol (1:1) liposome complexes and lung from untreated control are shown in Figs. 11A-11E. As demonstrated by the intense staining with the polyclonal anti-CFTR antibody, α-1468, the overwhelming majority of the airways were transfected with the human CFTR gene. See Figs. 11A, 11C and 11E. By visual inspection, essentially all the cells in transfected airways stain positively, demonstrating that the overwhelming majority of airway cells are transfected with the human CFTR gene *in vivo* with a single aerosol dose of pZN32 complexed to DDAB-cholesterol (1:1) liposomes. Representative sections are shown in Figure 11. There was no histologic evidence of lung damage, inflammation or edema present in any of the pZN32-DDAB:cholesterol (1:1) liposome-treated animals. pZN32-DDAB:cholesterol (1:1) liposome-treated and control animals could not be distinguished histologically. Significant expression of the human CFTR gene is

present in at least 50% of all the airways and at least 50% of all of the airway lining cells (by visual inspection) in mouse lungs for at least 60 days following a single aerosol dose of pZN32 complexed to DDAB-cholesterol (1:1) liposomes. Frozen sections of mouse lungs from control animals (Figs. 11B and 11D) do not show any detectable staining for CFTR, confirming that all the CFTR expression present in Fig. 11A, 11C and 11E is due to transfection of lung cells with the human CFTR gene.

Example 10

Demonstration of CAT Gene Expression in Lung and Liver After Intravenous Injection of Different CAT gene-Containing Plasmids

Lipid carrier:

DDAB:Chol= 1:1, stock 5mM in 5% dextrose in water.

Plasmids:

Plasmids are indicated below.

DNA-Lipid carrier Ratio:

cationic lipid: plasmid DNA = 1 nanomole: 1μg

Dose:

100μg DNA in 200ul volume injected intravenously by tail vein injection.

Mice:

ICR, female, 25 g

Procedure:

The animals were sacrificed 24 hours after injection. The tissue extraction procedure and CAT assay were as described in Example 12 except that the CAT assay was incubated for 3 hr at 37°C and 2.0mM paraoxon (Lai, C.-C. *et al*. *Carcinogenesis* 9:1295-1302 (1988)) was added to the liver samples. The results are shown in Figure 13. Lanes 1-12 are lung samples, lanes 13-24 are liver samples. Lanes 1, 2, 13, 14 are pZN51; lanes 3, 4, 15, 16 are pZN60; lanes 5, 6, 17, 18 are pZN61; lanes 7, 8, 19, 20 are pZN62; lanes 9, 10, 21, 22 are pZN63; and lanes 11, 12, 23, 24 are pZN27. pZN51, which does not contain an intron, is expressed as well as or better than plasmids containing an intron.

Example 11

Generalized versus tissue and cell type-specific CAT gene expression produced by iv injection of CMV-CAT-liposome or CFTR-CAT-liposome complexes, respectively.

Mouse: ICR female, 25 grams.

Liposome: DDAB:Cholesterol=1:1 SUV, 10mM in 5% dextrose in water.

Plasmid: 1) pZN27 or 2) pBE3.8CAT (see Chou *et al.*, *J. Biol Chem* 266:24471, 1991 for construction).

Procedure: Mice in groups of 3 received 1) no treatment, or a single iv tail vein injection of DDAB:CHOL liposomes complexed to 100 μg of 2) a 3.8 kb sequence of the 5' upstream region of the human CFTR gene fused to the CAT gene (pBE3.8CAT) or 3) pZN27. Mice were sacrificed 24 hours later and CAT activity assayed in lung, liver, spleen, lymph nodes, kidney and heart, as described in Example 12. Immunohistochemical analysis of lung section from each of the groups was performed as described in Example 11.

Results: Figure 14A-F CAT assay demonstrated that CMV-CAT produced significant CAT gene expression in the lung, liver, heart, spleen, lymph nodes and kidney, whereas CFTR-CAT produced lung-specific gene expression. Thus, the CMV promoter induces expression of a linked gene in a wide range of tissues, whereas the 5' flanking region of the human CFTR gene directs tissue-specific transgene expression after iv, liposome-based administration.

Immunohistochemical staining of frozen lung sections from these mice showed that iv injection of CMV-CAT-liposome complexes produced CAT gene expression in endothelial, alveolar and airway cells within the lung. In contrast, CFTR-CAT-liposome complexes produced CAT gene expression primarily in airway epithelial cells. (This approximates the pattern of endogenous CFTR gene expression in rat lung, as determined by *in situ* hybridization studies (Trezise and Buchwald, Nature, 353:434, 1991). This is the first demonstration that transgenes can be expressed within mouse lung in either a generalized or cell type-specific fashion after iv injection, depending on the regulatory element used. Results are shown in Figure 26A-E.

Example 12

High level, lung specific expression of a transgene complexed to cationic liposomes following aerosol administration

Animals.

Two month old, female, ICR mice were used in all experiments.

Preparation of plasmid DNA.

The chloramphenicol acetyltransferase (CAT) gene was used as a reporter to measure transgene expression levels (Gorman *et al.*, *Proc. Nat'l Acad Sci (USA)* (1982) 79: 6777-6781). The plasmid used contains the CAT gene fused to the human cytomegalovirus (CMV) immediate early promoter-enhancer element (pCIS-CAT). The plasmid was purified using alkaline lysis and ammonium acetate precipitation (Sambrook *et al.* (1989) supra), and the nucleic acid concentration measured by UV absorption at 260 nm. The CAT gene is not present in eukaryotic cells. Its product is an enzyme which catalyzes the transfer of acetyl groups from acetylCoA to the substrate chloramphenicol.

Preparation of cationic lipid carriers.

Lipid carriers were prepared as small unilamellar vesicles (approximately 100 nm in diameter) containing the cationic lipid DOTMA as DOTMA:DOPE (1:1 mole ratio). DOTMA is (N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (Syntex Corporation), and DOPE is the neutral lipid dioleoylphosphatidylethanolamine (Avanti Polar Lipids). Stock solutions of the lipids were dissolved in chloroform and stored under argon at -20°C. Lipids were mixed in a round-bottomed flask and evaporated to dryness on a rotary evaporator under reduced pressure. Double-distilled water was added to produce final lipid concentrations of 10mM each, and the resulting mix was sonicated for approximately 20 minutes in a bath sonicator (Laboratory Supplies, Hicksville, NY). The liposomes were stored under argon at 4°C until use.

Aerosol delivery of plasmid/lipid carrier complexes to mice.

Twelve mg of plasmid complexed to 24 μmols of DOTMA:DOPE (1:1) liposomes was aerosolized and administered to mice over two different aerosol periods on the same day. In order to prevent aggregation and precipitation of the oppositely charged components, the plasmid and the liposomes were diluted separately in sterile water prior to

mixing. Six mg of plasmid DNA and 12 μmols of DOTMA:DOPE (1:1) liposomes were each diluted to 8 ml with water and mixed. Four ml was then placed into each of two Acorn I nebulizers (Marquest, Englewood, CO), the animals placed into an Intox small animal exposure chamber (Albuquerque, NM), and an air flow rate of 4 L min^{-1} used to generate the aerosol. Approximately 90 minutes were required to aerosolize 4 ml. The animals were removed from the chamber for 1-2 hours and then the above procedure was repeated with a second 4 ml dose.

Radiometric Assay of CAT Activity.

Organs were dissected from animals sacrificed in a CO_2 chamber at periods from 1 to 21 days following aerosolization, washed in cold phosphate buffered saline (PBS), and homogenized using a hand-held tissue homogenizer in 250 mM Tris-HC1, pH. 7.5, containing 5 mM EDTA for lungs and spleen and 250 mM Tris-HC1, pH 7.5, containing 5 mM EDTA plus the protease inhibitors aprotinin, E-64, and leupeptic (Boehringer Mannheim) for liver, heart and kidneys. The inhibitors prevent degradation of acetylated chloramphenicol species generated during the assay, thereby allowing optimal detection of CAT expression.

Following homogenization of the tissue, cells were lysed by three freeze/thaw cycles, the lysate heated (65°C for 10 minutes), and centrifugated (16,000 x g, 2 minutes). The protein concentrations of the extracts were measured using a Coomassie blue-based assay (Bio-Rad). Protein concentrations were normalized and a volume of extract added to 10 μl of 100 mM acetylCoA (Sigma), 0.3 μCi of [^{14}C]-labelled chloramphenicol (Amersham), and distilled water to a final volume of 180 μl, and allowed to react at 37°C for 8-10 hours (Gorman *et al.* (1982) supra). Following the reaction, the acetylated and unacetylated chloramphenicol species were extracted with cold ethyl acetate, spotted on silica TLC plates, and developed with a chloroform:methanol (95:5v/v) solvent. The TLC plates were exposed to photographic film (Kodak X-OMAT) for one to three days and then read visually.

Preparation of Genomic DNA and Southern Hybridization.

Immediately following aerosolization, mice were sacrificed and their lungs removed. Genomic DNA was isolated and analyzed by Southern hybridization (Sambrook *et al.* (1989) supra) using a Hybond N^+ membrane (Amersham). A CAT probe was prepared from a 1.6

kb fragment of the CAT gene labelled with α-[^{32}P]dATP by random priming, which yielded a probe with an approximate specific activity of 2 x 10^9dpm/μg. After hybridization, the membrane was washed three times in 2xSSC, 0.1%SDS at 65°C for 20 minutes and exposed to film for 24 hours. In order to determine the approximate transfected CAT gene copy number, blots were also hybridized with a 1.1 kb BSU 36-1 single copy probe from a mouse factor VIII-A genomic clone (Levinson *et al.*, *Genomics* (1992) 13: 862-865). Relative amounts of the CAT plasmid deposited in individual mouse lungs were quantitated by phosphorimagining analysis using a Molecular dynamics 400A phosphorimaginer (Johnson *et al.*, *Electrophoresis* (1990) 11: 355-360). The amount of retained probe in each lane following hybridization with the CAT probe was normalized to the amount of DNA loaded per lane using the counts measured after hybridization with a Factor VIII-A single copy probe.

In *Situ* Immunochemical Staining for CAT enzyme.

At selected time points following aerosolization, mice were sacrificed and their lungs immediately removed. The lungs were slowly inflated with phosphate buffered saline (PBS) containing 33% by volume OCT (Miles, Inc.), placed in a tissue cassette filled with OCT, and frozen in 2-methylbutane chilled in a dry ice/ethanol bath. Cryosections were cut at 5 μm and collected onto salinized slides. CAT was detected after fixation of cryosections for 10 minutes in either 4% acetone or 2% paraformaldehyde in PBS containing 0.1% Tween 20 (PBST). All subsequent dilutions and washes were also done in PBST.

Following fixation, sections were washed three times (5 minutes each) then covered with 10% normal rabbit serum for 10 minutes at 20°C. The serum was replaced with diluted (1:500) rabbit polyclonal antibody against CAT (Drs. Parker Antin and David Standring, UCSF Medical Center). The antibody covered section was gently overlaid with a siliconized coverslip and incubated in a humid chamber at 4°C for 24 hours. Slides were then warmed to 20°C and washed three times. The presence of bound rabbit antibody against CAT was detected by covering sections with biotinylated, affinity purified, goat anti-rabbit antibody (Vector Laboratories) diluted 1:300 for 1 hour, followed by washing (3 x 10 minutes) and replacement with streptavidin labelled with alkaline phosphatase (Zymed, South San Francisco) for 20 minutes. Immobilized alkaline phosphatase was detected using AP-red (Zymed) as the chromogen, with endogenous alkaline phosphatase being inhibited with levamisole (Zymed). To control for potential spurious adherence of the streptavidin

conjugate to bronchiolar epithelium, some sections were treated with free avidin and biotin prior to application of the primary antibody. Other controls, run concurrently, included the use of normal rabbit serum in place of primary antibody and the use of lung tissue from untreated mice. Photo-microscopy was performed using Kodak Ektachrome 64T film X50 (Fig. 6 A,D) and X250 (Fig. 6 B,C,E,F).

Results

Initially, mice were exposed either to an aerosol generated from a solution containing 12 mg of a CMV-CAT expression plasmid alone or to an aerosol generated from a solution containing 12 mg of CMV-CAT complexed to 24 μmoles of DOTMA:DOPE (1:1) liposomes. Aerosols were administered to animals after they were placed individually in nose-out cones and inserted into an Intox small animal exposure chamber. The mice showed no apparent ill effects or respiratory distress either during or after aerosol exposure. Figure 7 shows the results of CAT assays from extracts of the lungs of mice sacrificed 72 hours following aerosol administration. Significant CAT gene expression was seen only in mice exposed to aerosolized DNA/lipid carrier complexes.

How long CAT protein was present in the lungs of mice and whether expression of the reporter gene was limited to the lung was also investigated. Despite inter-animal variation, high levels of CAT activity are present for at least 21 days following a single aerosol dose of DNA/lipid carrier complexes (Fig. 24A). No CAT activity was detectable in extracts from the heart, spleen, kidneys or liver of animals that showed high level expression in the lung (Fig. 24B), suggesting that transgene expression following aerosol delivery is restricted to the lung. This is consistent with prior observations showing that penetration of very high molecular weight substances through the respiratory epithelium of normal animals is very limited. Plasmid DNA/lipid carrier complexes have molecular weights greater than 10^6 daltons.

Although the small animal exposure chamber used in these experiments is designed to efficiently deliver a uniform aerosol dose to multiple animals up to 48 individual animals, we have observed significant variations in the level of CAT activity in the lungs of mice within a single experiment. One possible explanation for this variability is that the amount of DNA/liposome complex deposited in the lungs of mice is not uniform. In order to test this hypothesis, initial lung deposition of liposomes was measured using fluorescence analysis and initial lung deposition of DNA was measured using Southern blot analysis.

Aerosolized cationic liposomes alone or DNA/liposome alone or DNA/liposome complexes containing 0.5 mole percent of a fluorescently labelled lipid, rhodamine-phosphatidylethanolamine, were administered to mice. Immediately following aerosolization, the animals were sacrificed and their lungs removed, homogenized and rhodamine fluorescence measured using a fluorimeter. The recovered fluorescence per animal was 0.06% $\pm$ 0.02 (S.D.) of the total amount aerosolized. This suggests that less than 10 μg out of the 12 mg of DNA aerosolized per experiment was actually deposited in the lung. In addition, there was no significant difference in lipid deposition between animals receiving liposomes alone and those receiving the DNA/liposomes complexes. Since it is possible that a disruption of the complex could have occurred during nebulization, the amount of CAT gene deposited during aerosolization (Fig. 25) was also assessed. Immediately following aerosol delivery of DNA/lipid carrier complexes, mice were sacrificed and total lung DNA prepared. Southern blots were probed with $\alpha[^{32}P]$-labelled CAT gene. Labelled bands were scanned and demonstrated less than a 4-fold difference in plasmid deposition between animals in the same experiment (Fig. 25). These results suggest that the mouse to mouse variation in CAT gene levels following aerosol delivery (up to ten-fold) is not only a function of the amount of complex initially deposited in the lung, but also may reflect differences in the site of uptake, rate of lung clearance, and/or variation in the ability of different lung cell types to express the transgene.

To determine the types and percentage of lung cells which were transfected *in vivo*, lungs of mice sacrificed 72 hours following exposure to an aerosol containing DNA/liposome complexes were cryosectioned, probed with a polyclonal anti-CAT antibody and counterstained to detect intracellular CAT protein (Fig. 22). Lung sections taken from DNA/lipid carrier treated mice had a diffuse immunostaining pattern involving bronchiolar and alveolar components. The bronchiolar epithelial cytoplasm stained with greatest intensity and uniformity. CAT antigen was detected (as demonstrated by red staining) in nearly all conducting airways with only rare individual or 2-3 cell clusters not staining (Fig. 22A, 22B). The diffuse alveolar pattern was due to moderately intense staining of the majority of alveolar lining cells (Fig. 22C). These areas occasionally faded into small, randomly scattered regions where lining cell staining was faint. Focal, intense staining (arrows) occurred in the cytoplasm of scattered, individual, alveolar lining cells (Fig. 22C). Controls included substitution of the primary antibody with normal rabbit serum (Fig. 22D) and use of lung sections from untreated animals (Fig. 22E, 22F). Immunostaining was not

detectable in either of the control preparations. Examination of multiple sections of lung from treated and control mice demonstrated no significant lesions which would indicate adverse effects of the aerosol treatment.

As shown by the above results, a single aerosol dose of an expression liposome, containing a gene of interest, complexed to cationic liposomes transfects the majority of the cells lining both the conducting airways and the alveoli of the lung, the gene product is present in the lung for at least 60 days, the expression appears to be lung-specific, and there is no histological evidence of damage following exposure. Thus, the aerosolized cationic liposomes mediate efficient transfection of non-dividing as well as dividing cells. This is important because many airway epithelial cells are well differentiated and divide slowly or not at all. The lipid carriers appear to be both well tolerated and non-immunogenic. Furthermore, the appearance, behavior and life span of mice treated with either aerosolized or injected pZN32: DDAB-cholesterol (1:1) complexes appear normal and are indistinguishable from untreated, normal control animals, demonstrating the lack of toxicity of these carrier constructs, and the overexpression of the human CFTR gene in mammals. Additionally, the effects of repeated aerosol administration of the DNA/liposome complexes is effective and is non-toxic. The cationic liposome-mediated DNA delivery by aerosol provides high level, lung-specific transgene expression *in vivo*.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS

1. A composition for use in a method of treatment or therapy of a disease in the human or animal body, said disease relating to an insufficient amount of endogenous production of wild-type cystic fibrosis transmembrane conductance regulator, said composition comprising a transcription cassette or an expression cassette in a pharmaceutically acceptable carrier or diluent wherein said transcription cassette or said expression cassette comprises a DNA sequence capable of producing a transcription product of an open reading frame encoding a molecule having wild-type cystic fibrosis transmembrane conductance regulator activity in cells transfected with said transcription cassette or said expression cassette.

2. The composition for use according to Claim 1, wherein said transcription cassette or expression cassette is associated with a cationic lipid carrier.

3. The composition for use according to Claim 2, wherein said composition is nebulized following said association of said transcription cassette or said expression cassette and said cationic lipid carrier.

4. The composition for use according to Claim 2 or 3, wherein said cells transfected are distal airway cells or airway submucosal cells.

5. The composition for use according to Claim 2 or 3, wherein said cells are tracheal cells.

6. The composition for use according to Claim 4 or 5, wherein from 20 to 100 percent of said cells are transfected.

7. The composition for use according to Claim 1 or 2, wherein said DNA sequence comprises an inducible promoter.

8. The composition for use according to Claim 1 or 2, wherein said inducible promoter is a cell specific promoter, a tissue specific promoter, or a hormone responsive promoter.

9. The composition for use according to any one of Claims 1, 2 or 8, wherein said cell specific promoter promoter or siad tissue specific promoter is a promoter from a cystic fibrosis transmembrane conductance regulator gene.

10. The composition for use according to any one of Claims 1, 2, 8 or 9, wherein said DNA sequence comprises an SV40 enhancer element whereby transcription from said promoter is enhanced.

11. The composition for use according to any one of claims 2 to 10, wherein said cationic lipid carrier comprises a lipid selected from the group consisting of N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA); dimethyl dioctadecyl ammonium bromide (DDAB); 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP); lysinylphosphatidyl-ethanolamine (L-PE); distearoylphosphatidylethanolamine (DOPE); and cholesterol (Chol).

12. The composition for use according to any one of Claims 2 to 10, wherein said cationic lipid carrier comprises cholesterol and a lipid selected from the group consisting of N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA); dimethyldioctadecylammoniumbromide (DDAB); 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP); and lysinylphosphatidyl-ethanolamine (L-PE).

13. The composition for use according to any one of Claims 2 to 10, wherein said cationic lipid carrier comprises distearoyl-phosphatidylethanolamine (DOPE) and a lipid selected from the group consisting of N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA); dimethyldioctadecylammoniumbromide (DDAB); 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP); and lysinylphosphatidyl-ethanolamine (L-PE).

14. The composition for use according to any one of Claims 2 to 10, wherein said cationic lipid carriers are small unilamellar vesicles.

15. The composition for use according to Claim 14, wherein said small unilamellar vesicles comprise (a) distearoyl-phosphatidylethanolamine (DOPE) and N[1-2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA) or (b) dimethyldioctadecyl-ammoniumbromide (DDAB) and cholesterol.

16. The composition for use according to any one of Claims 2 to 10, wherein said transcription cassettes or expression cassettes and said cationic lipid carriers are present in said mixture in a ratio in the range of from about 1:1 to 1:2 micrograms DNA to nanomoles of cationic lipid.

17. A kit for use in a method of treatment or therapy according to any one of Claims 2 to 16, said kit comprising in combination:

a container containing said transciption cassettes or expression cassettes;

another container containing a specific amount of cationic lipid carriers; and

instructions.

18. The composition for use according to any one of Claims 1 to 16, wherein said open reading frame is intron-free.

19. The composition for use according to any one of Claims 1 to 16, wherein said DNA sequence comprises an intron 5' to said open reading frame.

20. The composition for use according to any one of Claims 1 to 16, wherein said DNA sequence comprises an expanded intron 3' to said open reading frame.

21. The composition for use according to Claim 1 or 2, wherein said method of treatment or therapy is intraoral or intranasal administration following nebulization of said composition.

22. The composition for use according to Claim 1 or 2, wherein said method of treatment or therapy is intravenous injection of said composition.

23. The composition for use according to Claim 1 or 2, wherein said method of treatment or therapy is a combination of intraoral or intranasal administration following nebulization of said composition and intravenous injection of said composition.

24. The composition for use according to Claim 9, wherein cystic fibrosis transmembrane conductance regulator (CFTR) gene is a human wild-type gene.

25. The composition for use according to any one of Claims 1 to 24, wherein said open reading frame encodes a molecule having the biological activity of wild-type human CFTR.

26. The composition for use according to any one of Claims 1 to 24, wherein said open reading frame is from a wild-type human CFTR gene.

27. The composition for use according to any one of Claims 9, or 24-26, wherein said DNA sequence comprises one or more enhancer elements from a gene other than a CFTR gene.

28. A kit for use in a method of treatment or therapy according to any one of Claims 2 to 27, said kit comprising in combination:

a container containing said transciption cassettes or expression cassettes;

another container containing a specific amount of cationic lipid carriers; and

instructions.

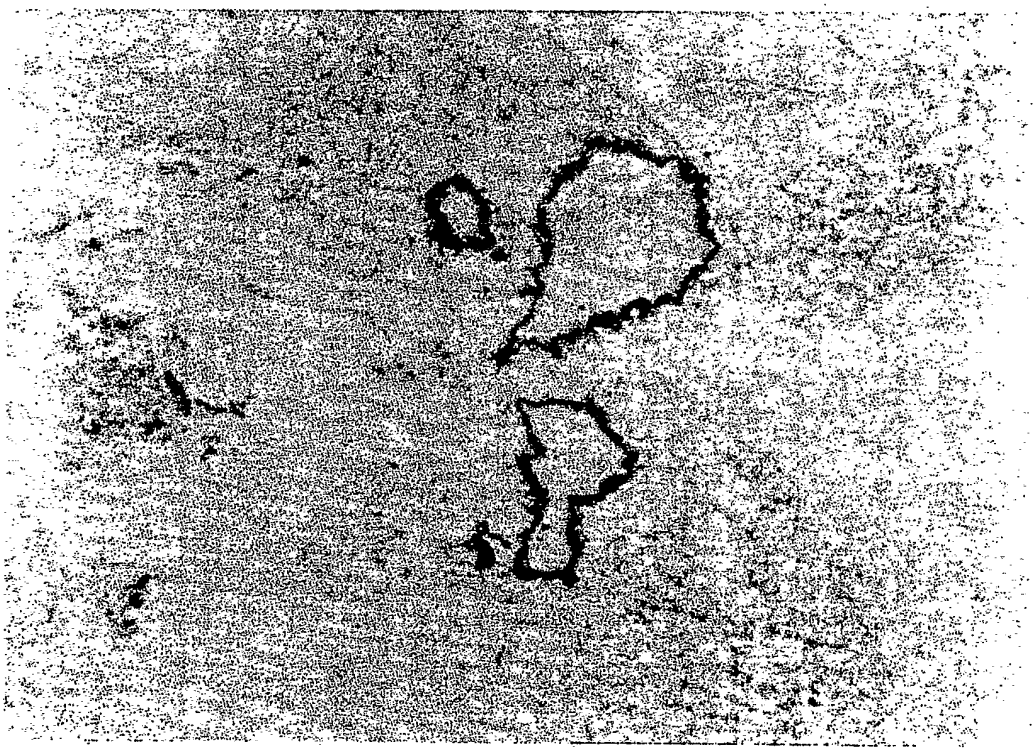


FIGURE 1A

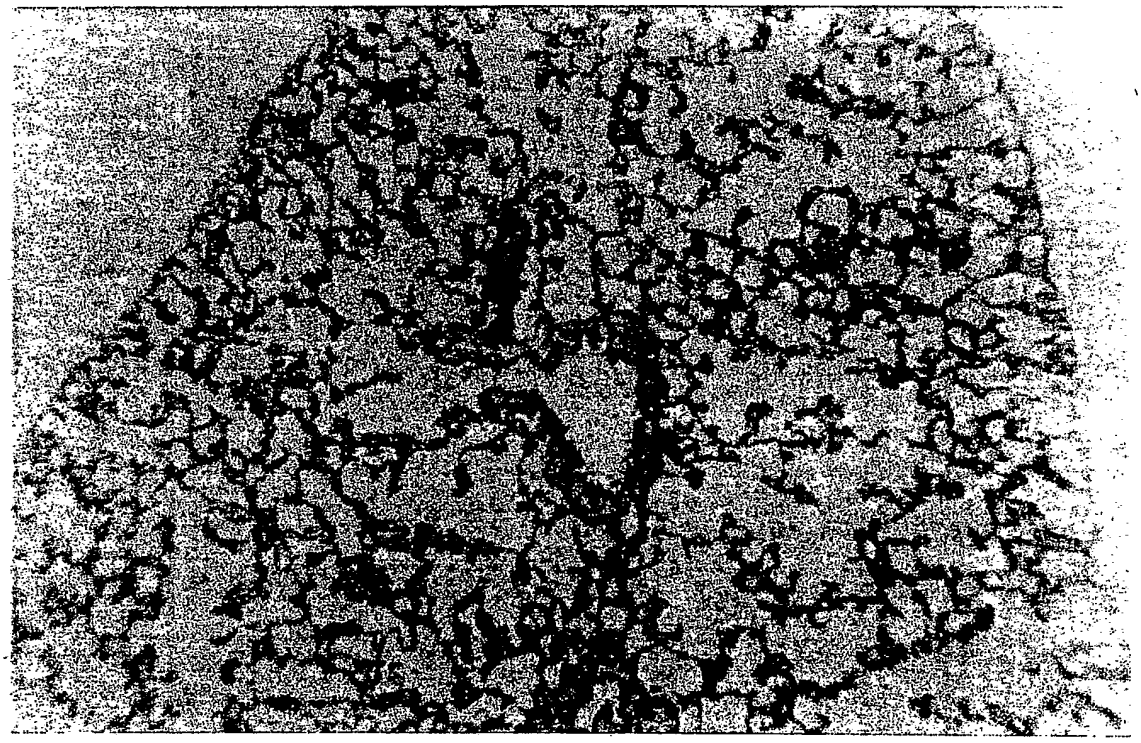


FIGURE 1B

FIGURE 1C

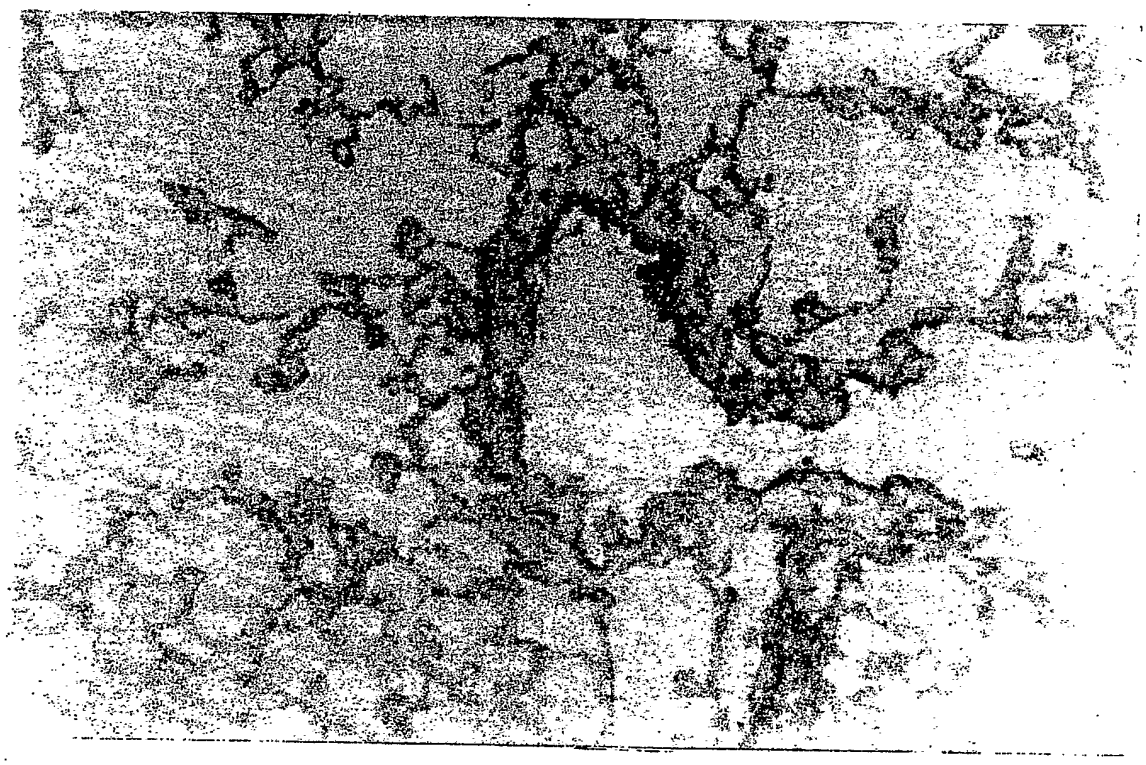


FIGURE 1D

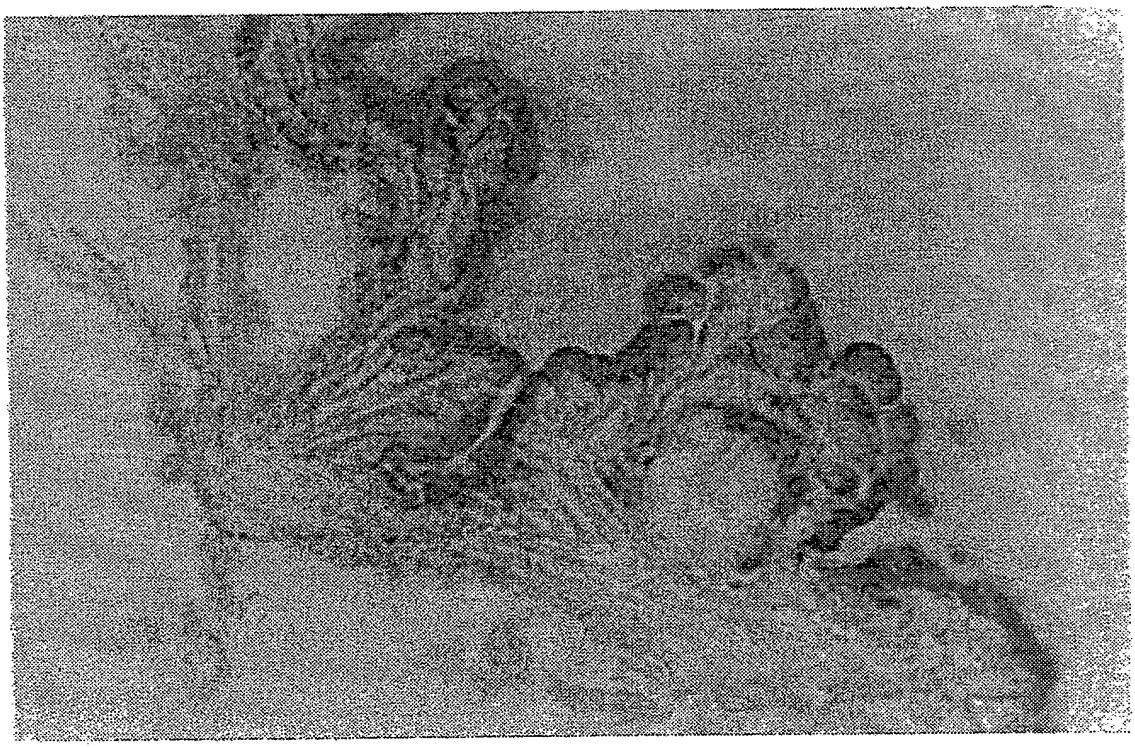


FIGURE 1E

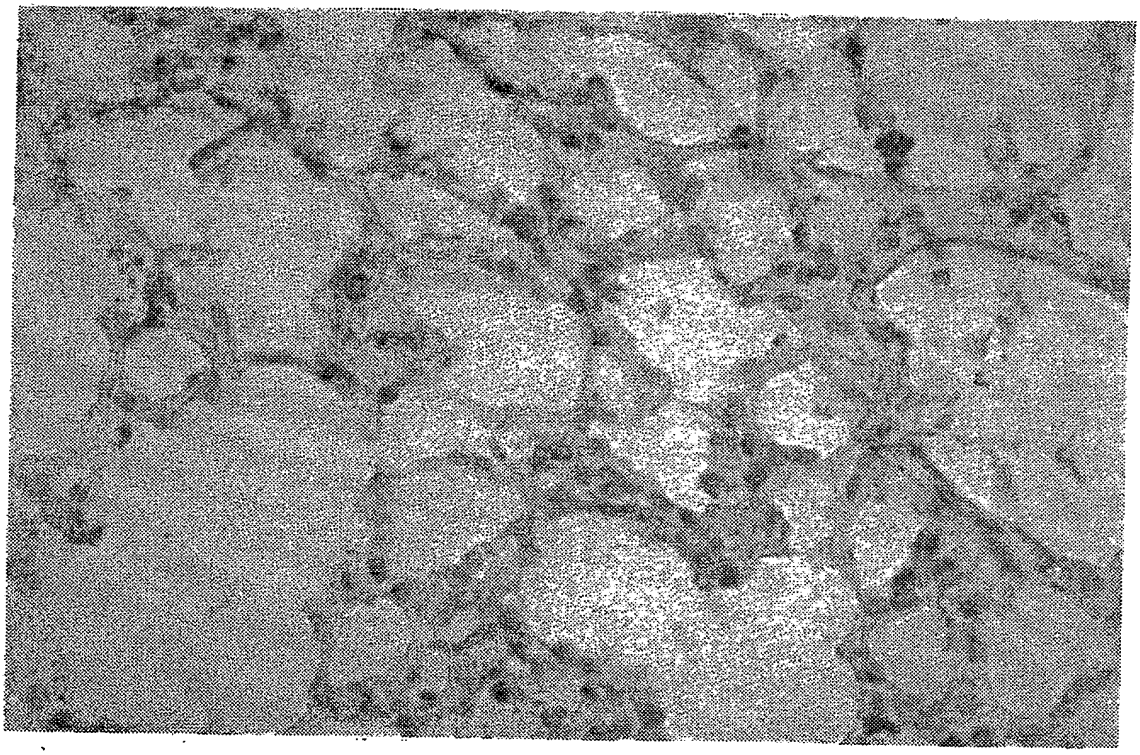


FIGURE 2A

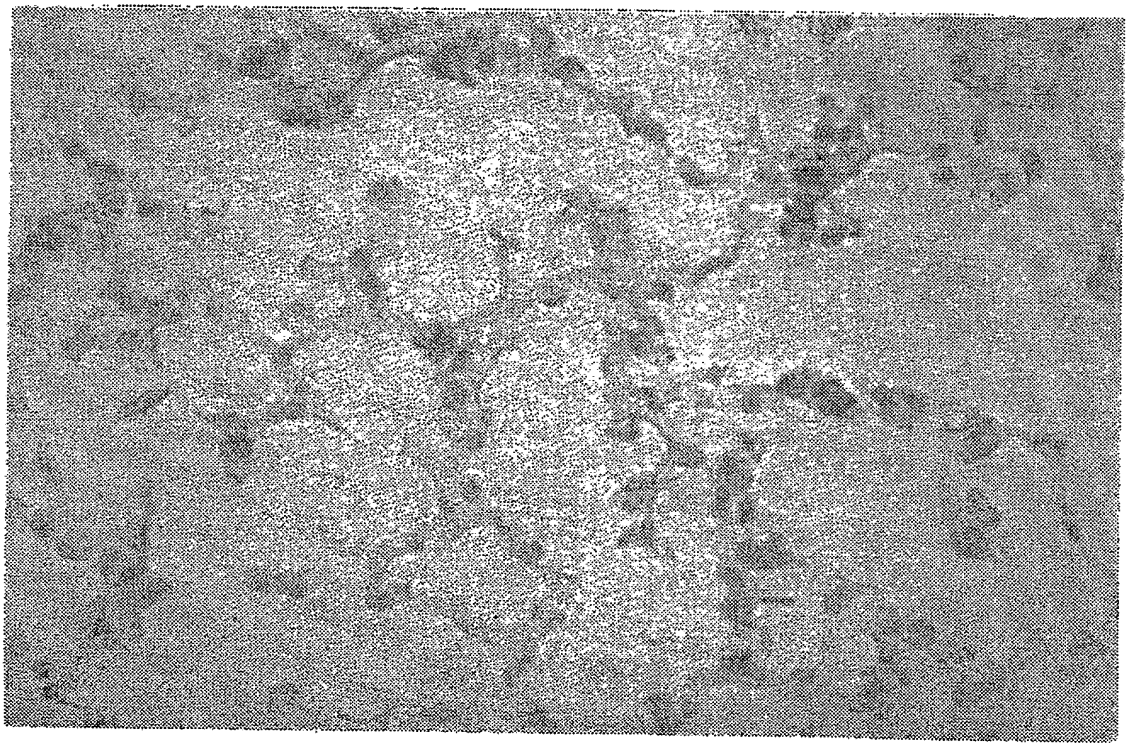


FIGURE 2B

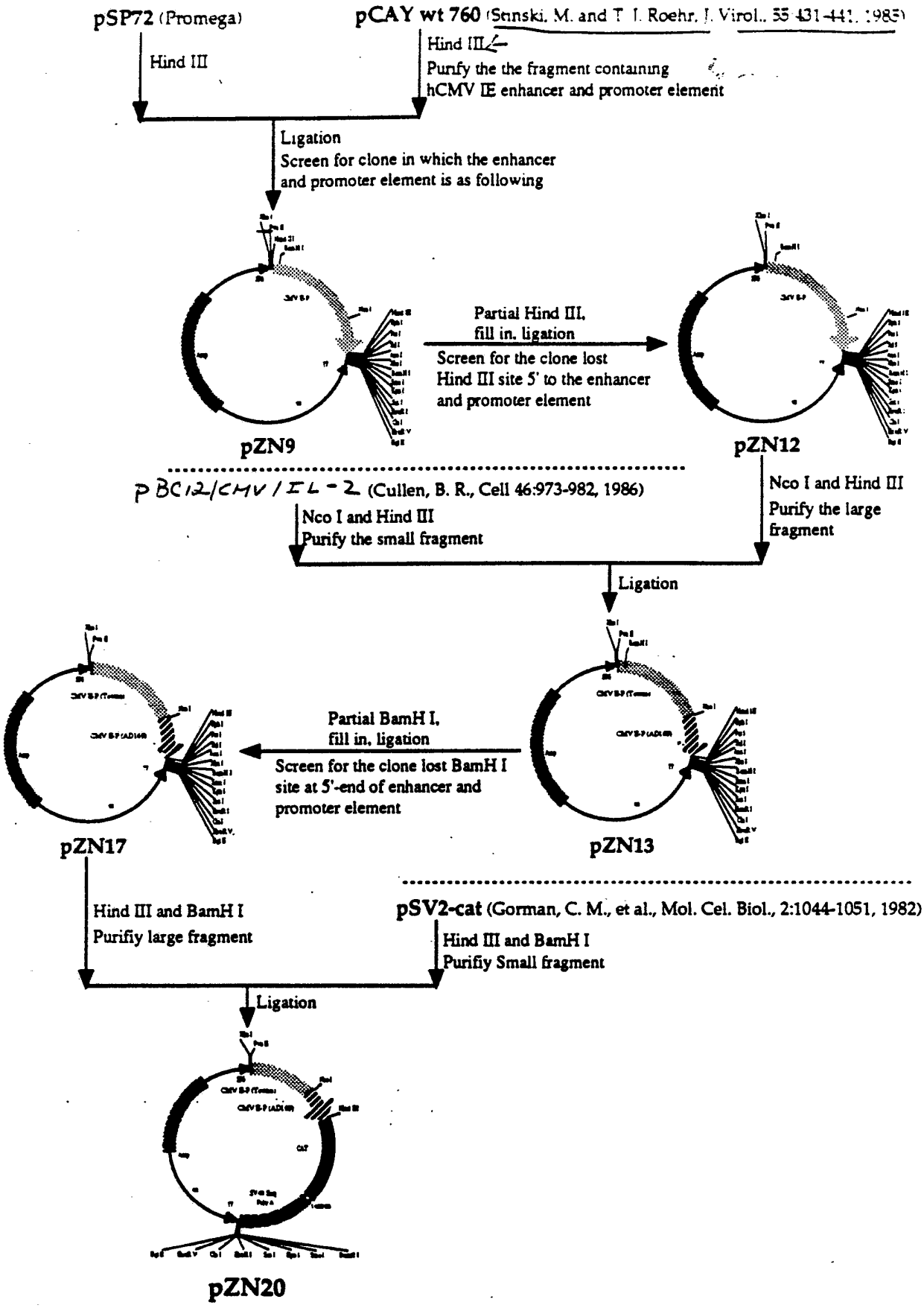

pSP72 (Promega)
Hind III
pCAY wt 760 (Stinski, M. and T. J. Roehr, J. Virol., 55:431-441, 1985)
Hind III
Purify the the fragment containing hCMV IE enhancer and promoter element
Ligation
Screen for clone in which the enhancer and promoter element is as following
pZN9
Partial Hind III, fill in, ligation
Screen for the clone lost Hind III site 5' to the enhancer and promoter element
pZN12
pBC12/CMV/IL-2 (Cullen, B. R., Cell 46:973-982, 1986)
Nco I and Hind III
Purify the small fragment
Nco I and Hind III
Purify the large fragment
Ligation
pZN17
Partial BamH I, fill in, ligation
Screen for the clone lost BamH I site at 5'-end of enhancer and promoter element
pZN13
Hind III and BamH I
Purifiy large fragment
pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)
Hind III and BamH I
Purifiy Small fragment
Ligation
pZN20

FIGURE 3

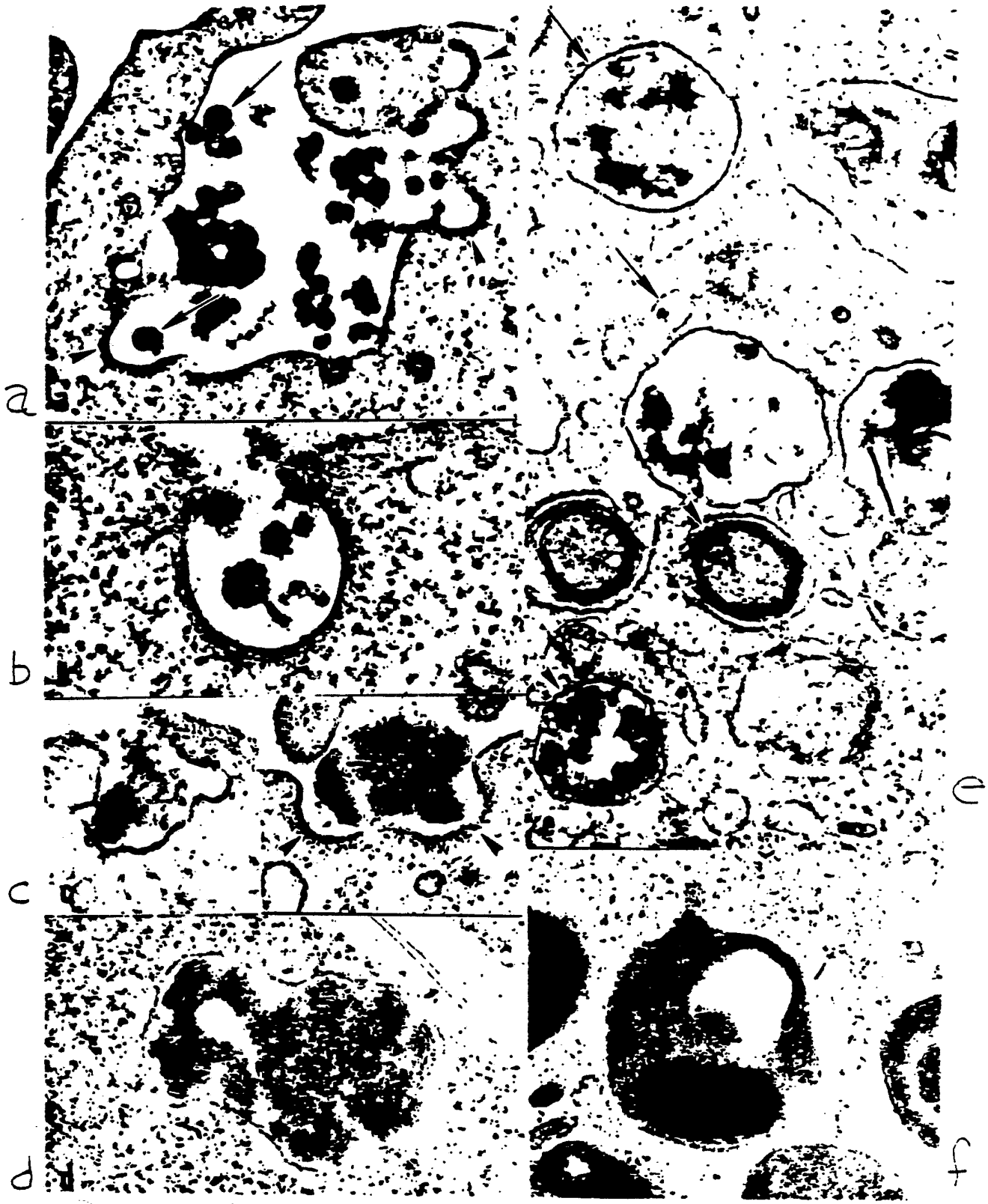


FIGURE 4

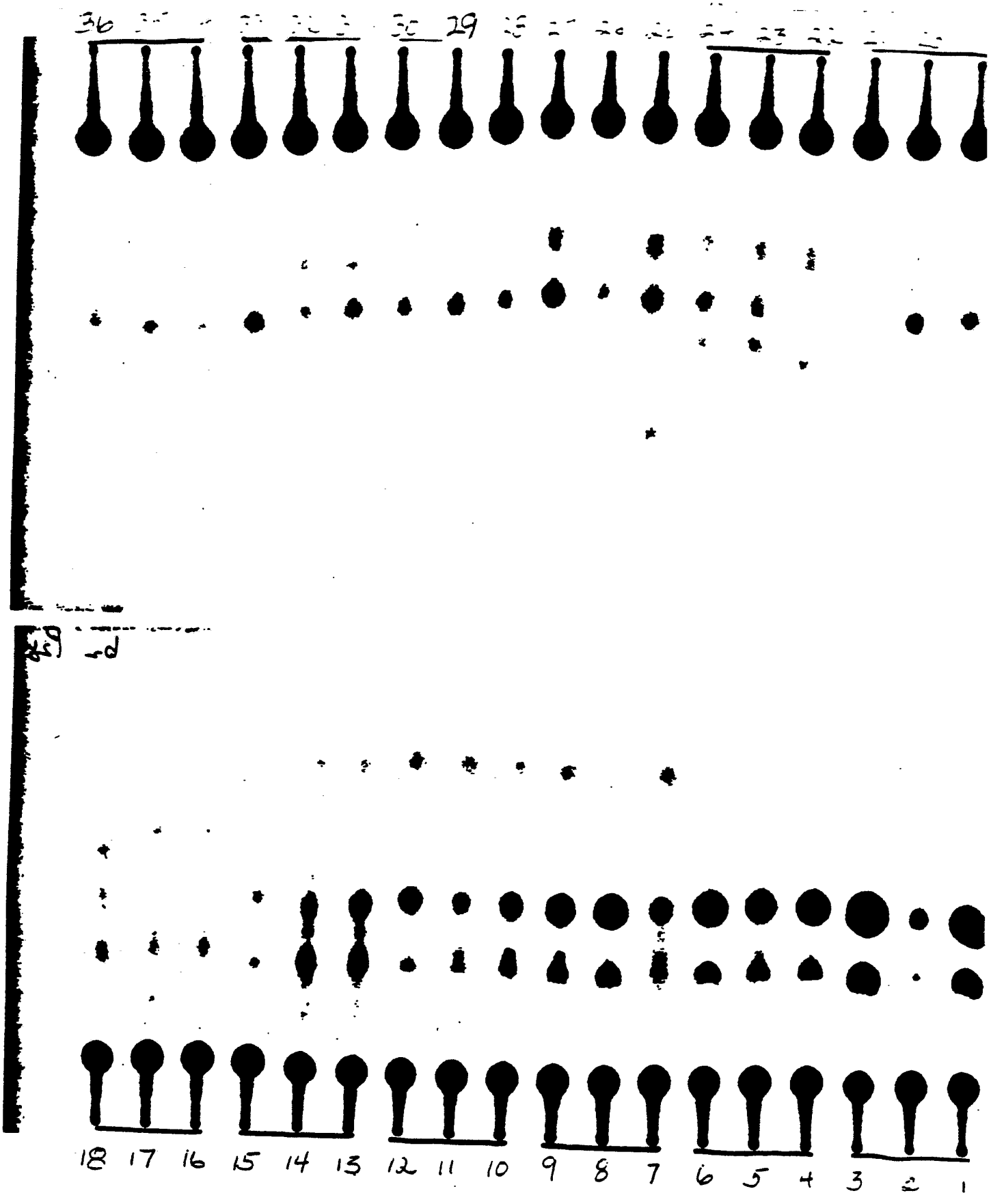


FIGURE 5

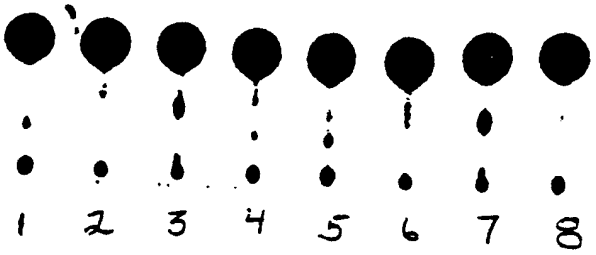


FIGURE 9A

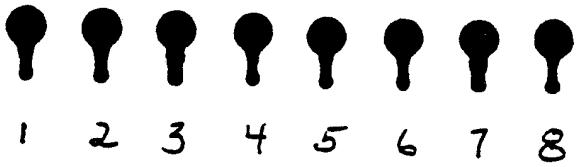


FIGURE 6

pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)

BstY I, fill in

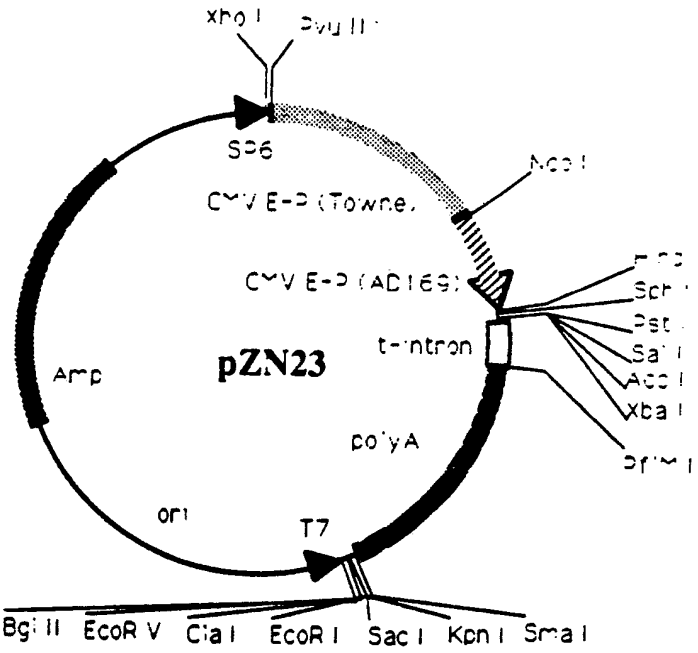
Gel purify fragment containing t-intron and polyA site (847bp)

pZN17

BamH I, fill in

Gel purify the vector

Ligation



pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)

Hind III+Ban I, fill in

Gel purify the CAT containing fragment (716bp)

PflM I, Blunt-ended by Klenow

Gel purify the vector

Ligation

Screen for clone containing the CAT gene in sence orientation

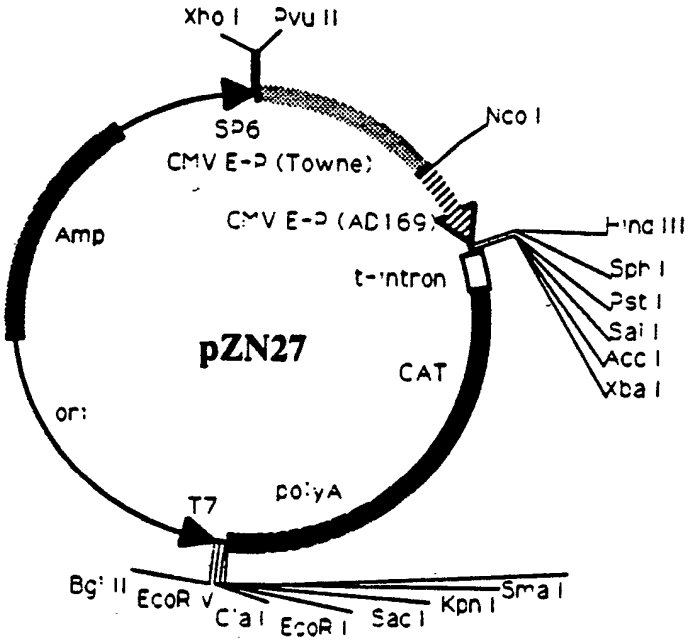


FIGURE 7

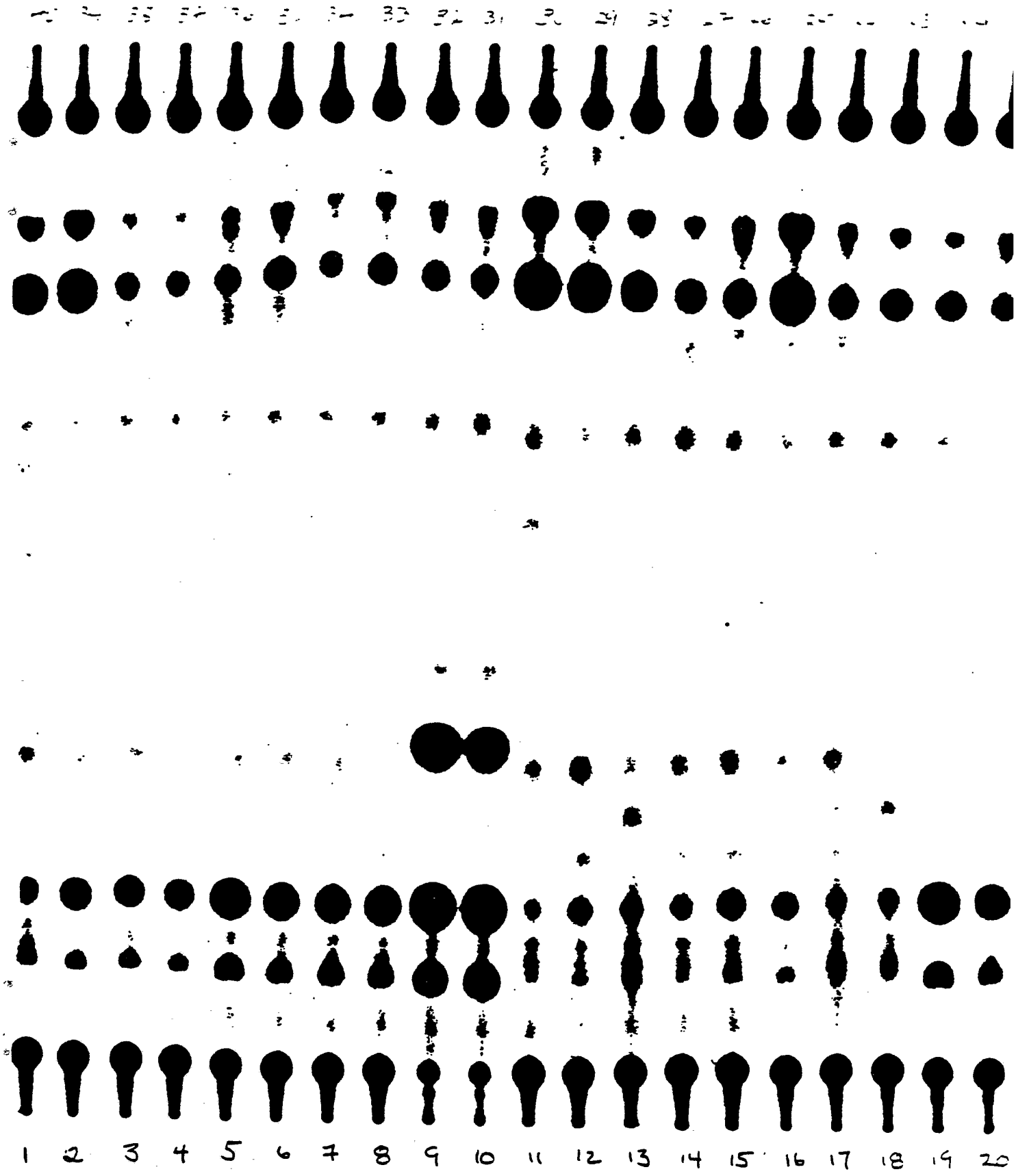


FIGURE 8A.

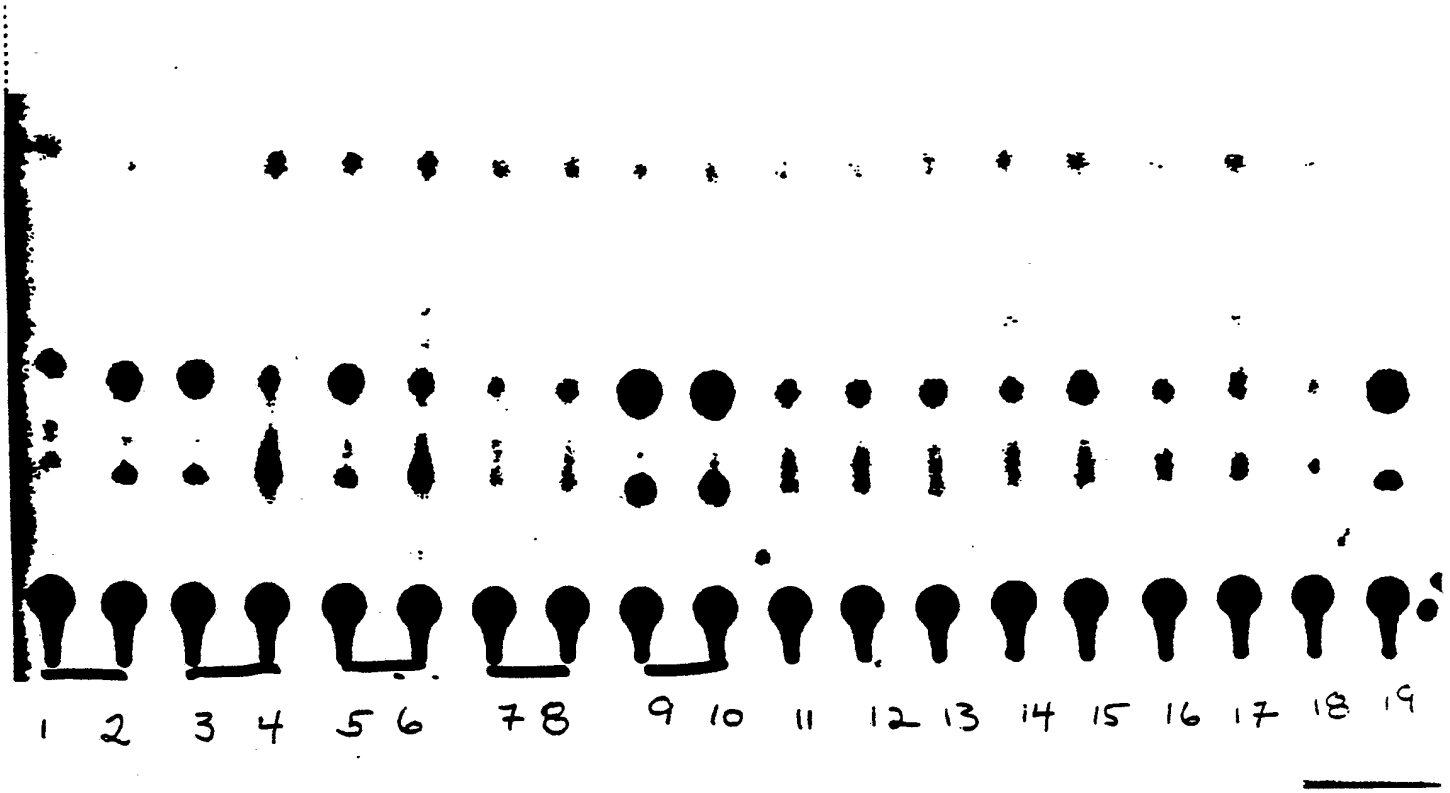


FIGURE 8B

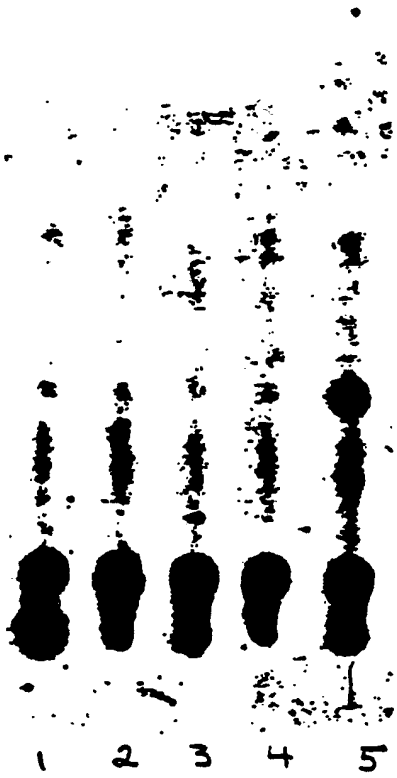


FIGURE 9

pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)

Pst I, fill in, gel purify CFTR cDNA insert (~4.7kb)

pZN29

Sma I

Ligation
Screen for clone containing insert
in the sense orietation

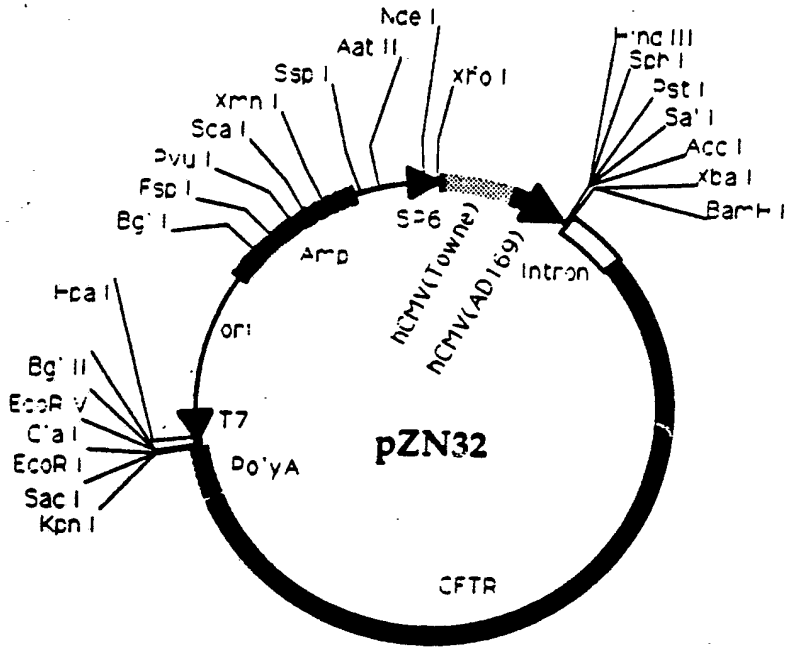


FIGURE 10

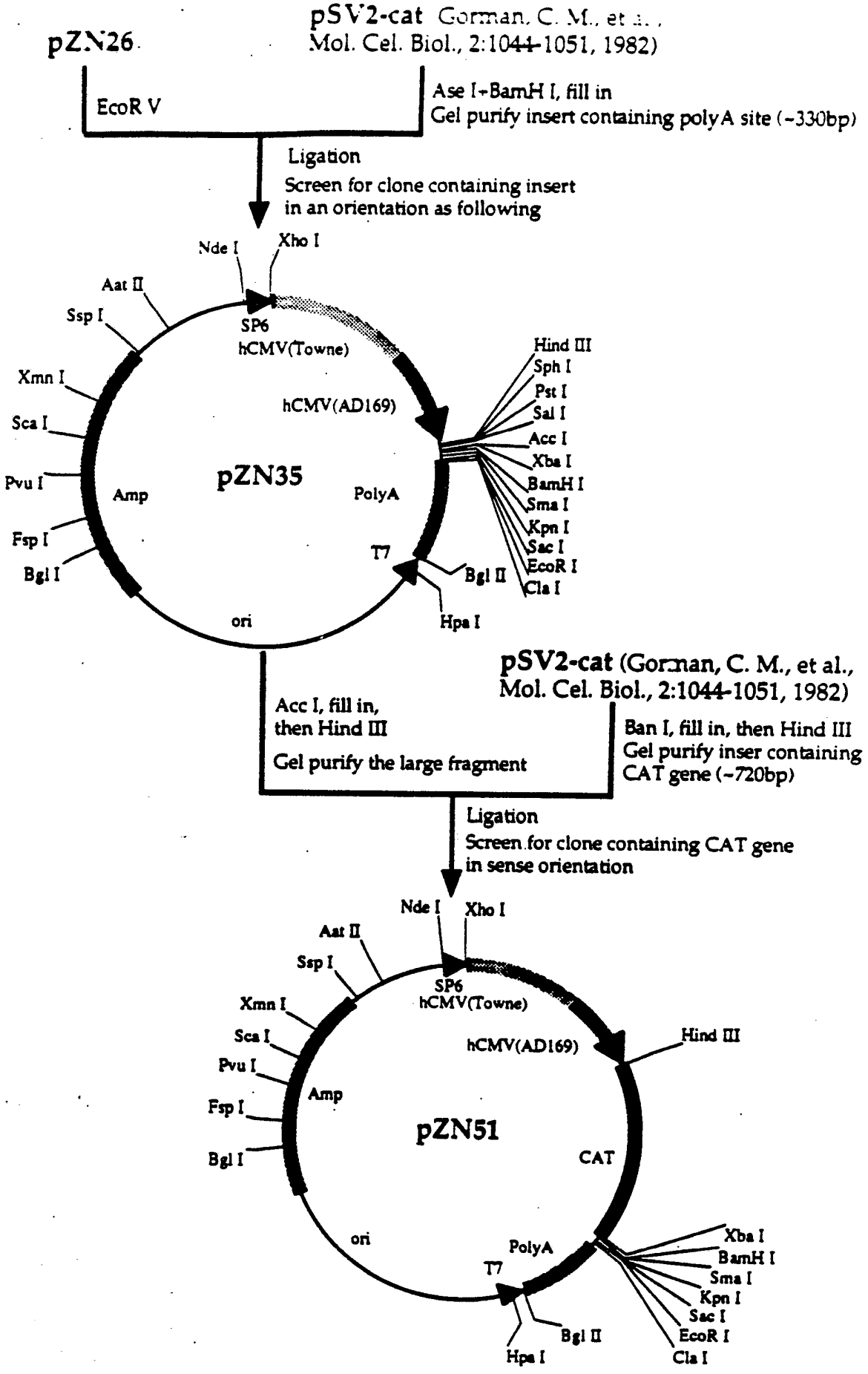

pZN26
pSV2-cat Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)
EcoR V
Ase I+BamH I, fill in
Gel purify insert containing polyA site (~330bp)
Ligation
Screen for clone containing insert in an orientation as following
Nde I
Xho I
Aat II
Ssp I
SP6
hCMV(Towne)
Xmn I
hCMV(AD169)
Sca I
pZN35
Pvu I
Amp
PolyA
Fsp I
T7
Bgl I
Bgl II
ori
Hpa I
Hind III
Sph I
Pst I
Sal I
Acc I
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
Cla I
pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)
Acc I, fill in, then Hind III
Gel purify the large fragment
Ban I, fill in, then Hind III
Gel purify inser containing CAT gene (~720bp)
Ligation
Screen for clone containing CAT gene in sense orientation
Nde I
Xho I
Aat II
Ssp I
SP6
hCMV(Towne)
Xmn I
Sca I
hCMV(AD169)
Hind III
Pvu I
Amp
Fsp I
pZN51
Bgl I
CAT
ori
PolyA
T7
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
Bgl II
Hpa I
Cla I

FIGURE 11

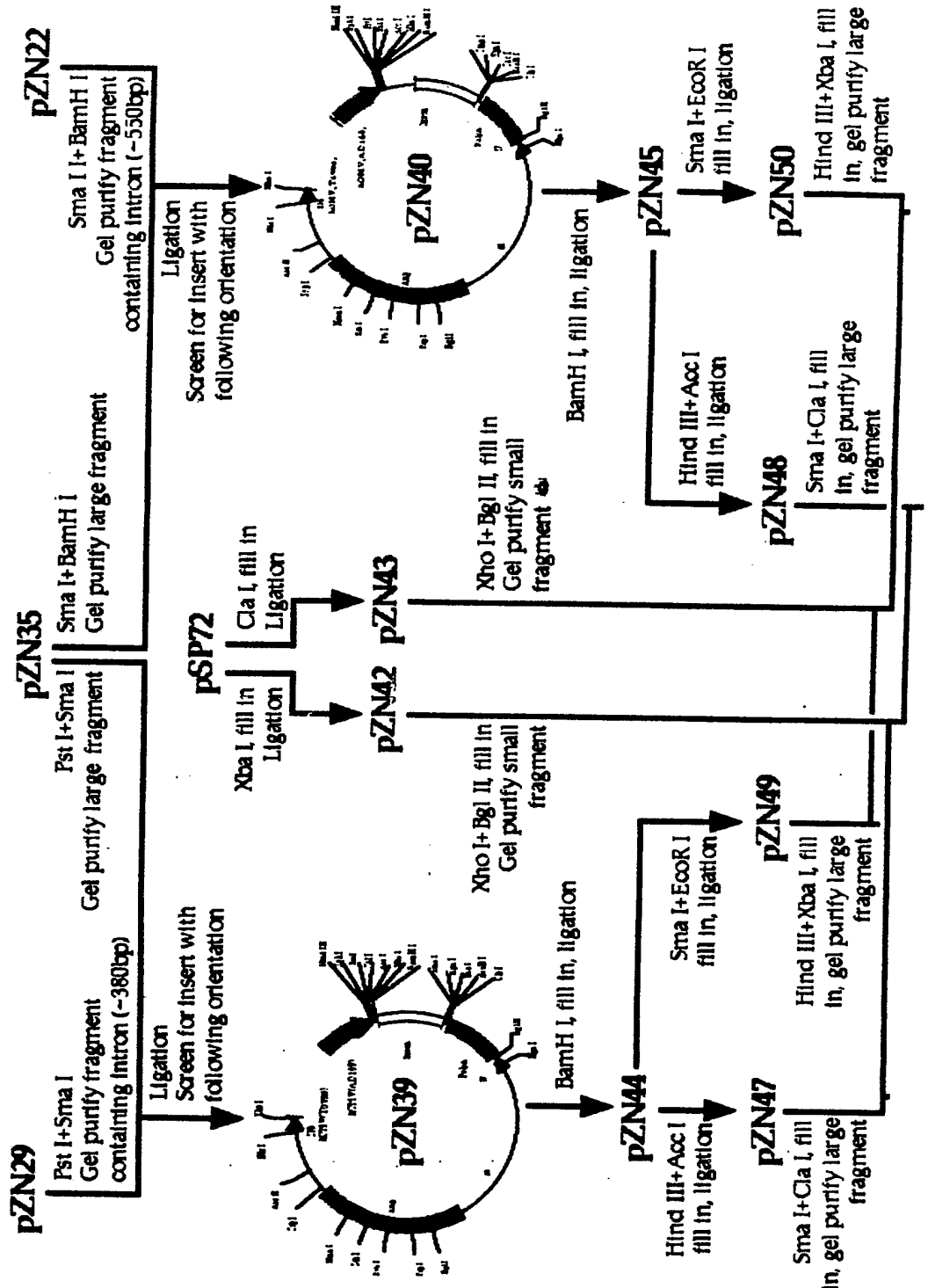

pZN22
Sma I+BamH I
Gel purify fragment
containing Intron (~550bp)
pZN35
Sma I+BamH I
Gel purify large fragment
Pst I+Sma I
Gel purify large fragment
pZN29
Pst I+Sma I
Gel purify fragment
containing Intron (~380bp)
Ligation
Screen for Insert with
following orientation
pZN40
pZN39
pSP72
Cla I, fill in
Ligation
Xba I, fill in
Ligation
pZN43
pZN42
Xho I+Bgl II, fill in
Gel purify small
fragment
BamH I, fill in, ligation
pZN45
Sma I+EcoR I
fill in, ligation
Hind III+Acc I
fill in, ligation
pZN48
Sma I+Cla I, fill
in, gel purify large
fragment
pZN50
Hind III+Xba I, fill
in, gel purify large
fragment
pZN44
pZN47
pZN49

FIGURE 12A

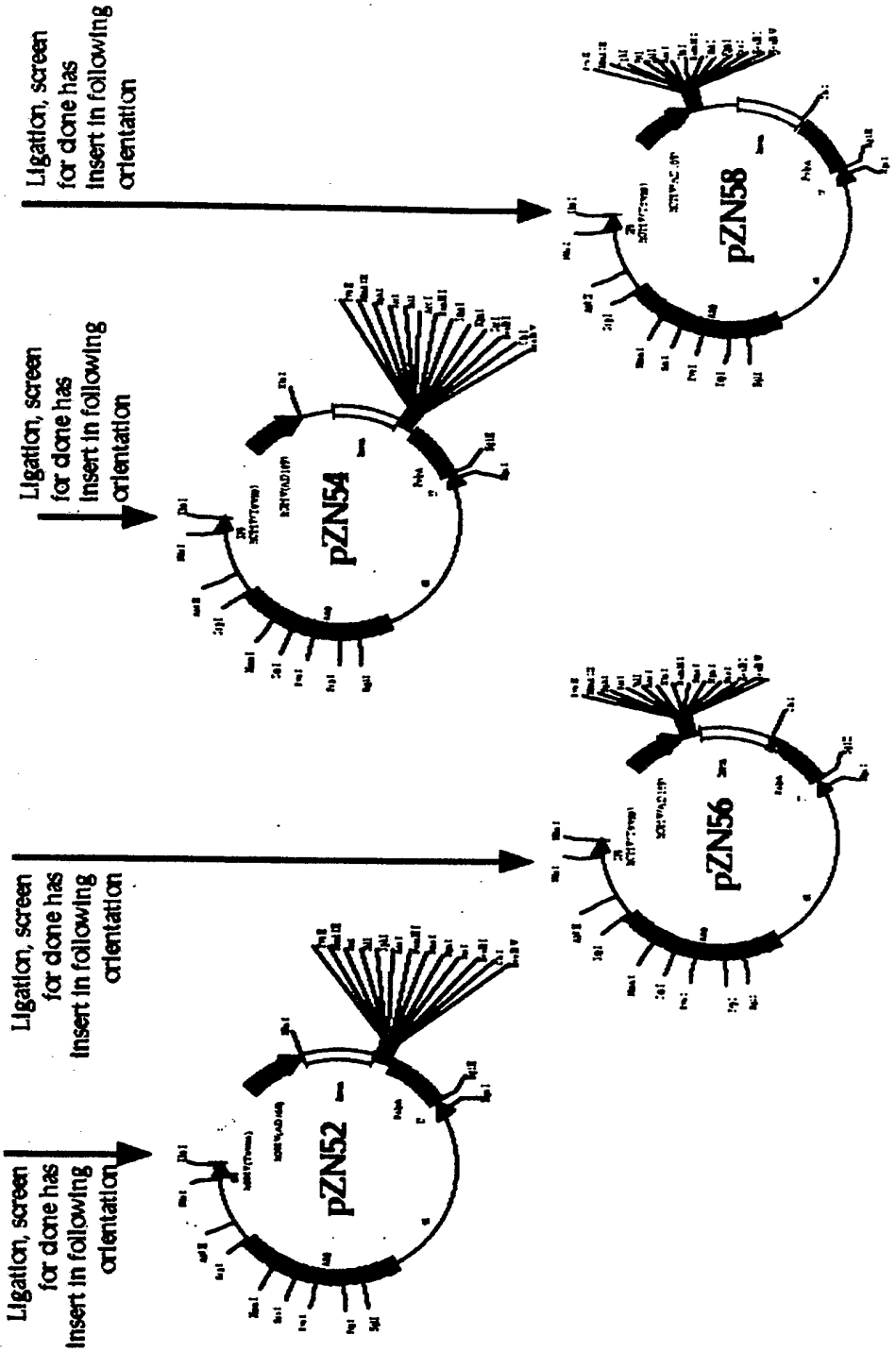

Ligation, screen for done has insert in following orientation
pZN58
Ligation, screen for done has insert in following orientation
pZN54
Ligation, screen for done has insert in following orientation
pZN56
Ligation, screen for done has insert in following orientation
pZN52

FIGURE 12B

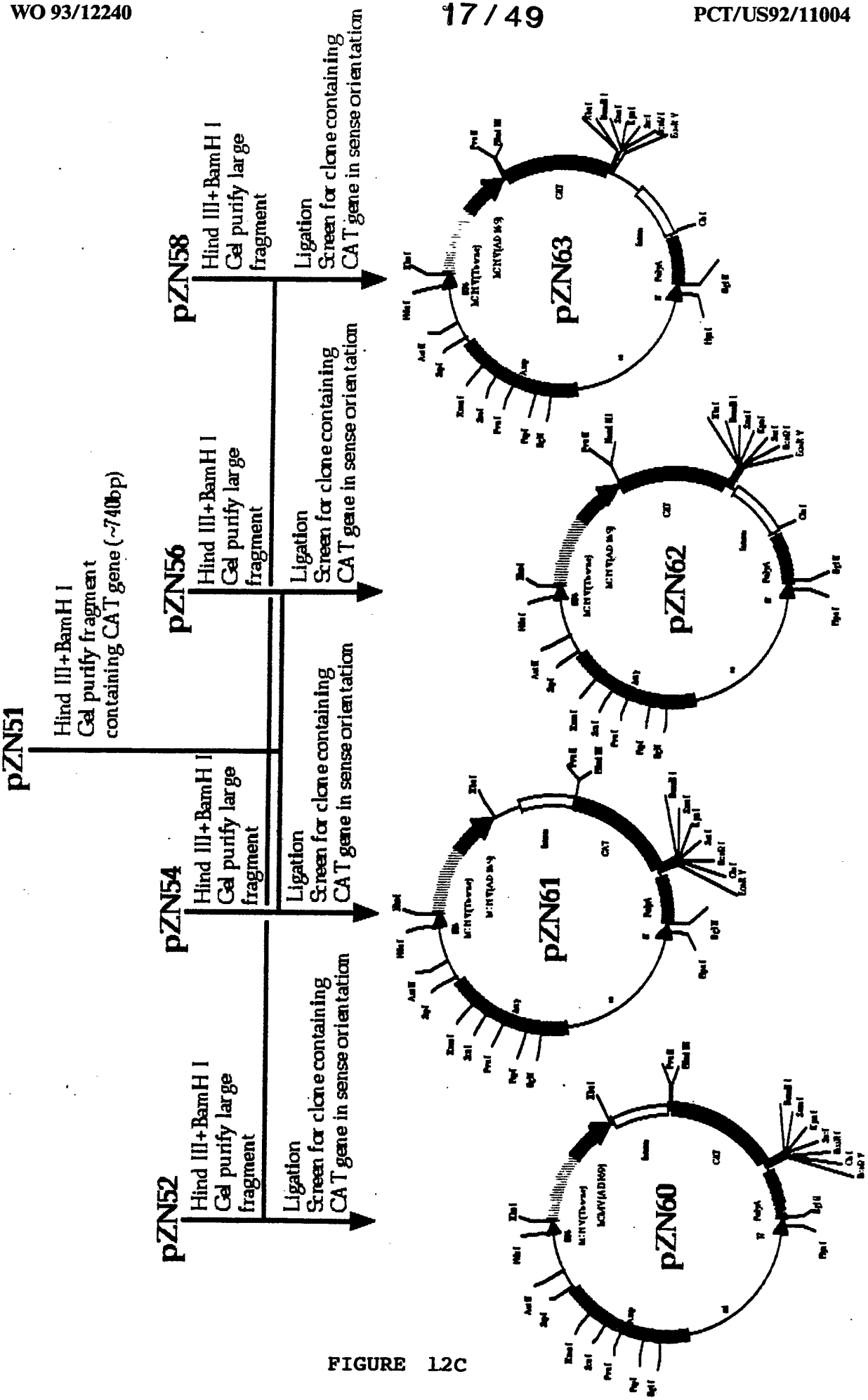

pZN51
Hind III+BamH I
Gel purify fragment
containing CAT gene (~740bp)
pZN52
Hind III+BamH I
Gel purify large
fragment
Ligation
Screen for clone containing
CAT gene in sense orientation
pZN54
Hind III+BamH I
Gel purify large
fragment
Ligation
Screen for clone containing
CAT gene in sense orientation
pZN56
Hind III+BamH I
Gel purify large
fragment
Ligation
Screen for clone containing
CAT gene in sense orientation
pZN58
Hind III+BamH I
Gel purify large
fragment
Ligation
Screen for clone containing
CAT gene in sense orientation
pZN60
pZN61
pZN62
pZN63

FIGURE 12C

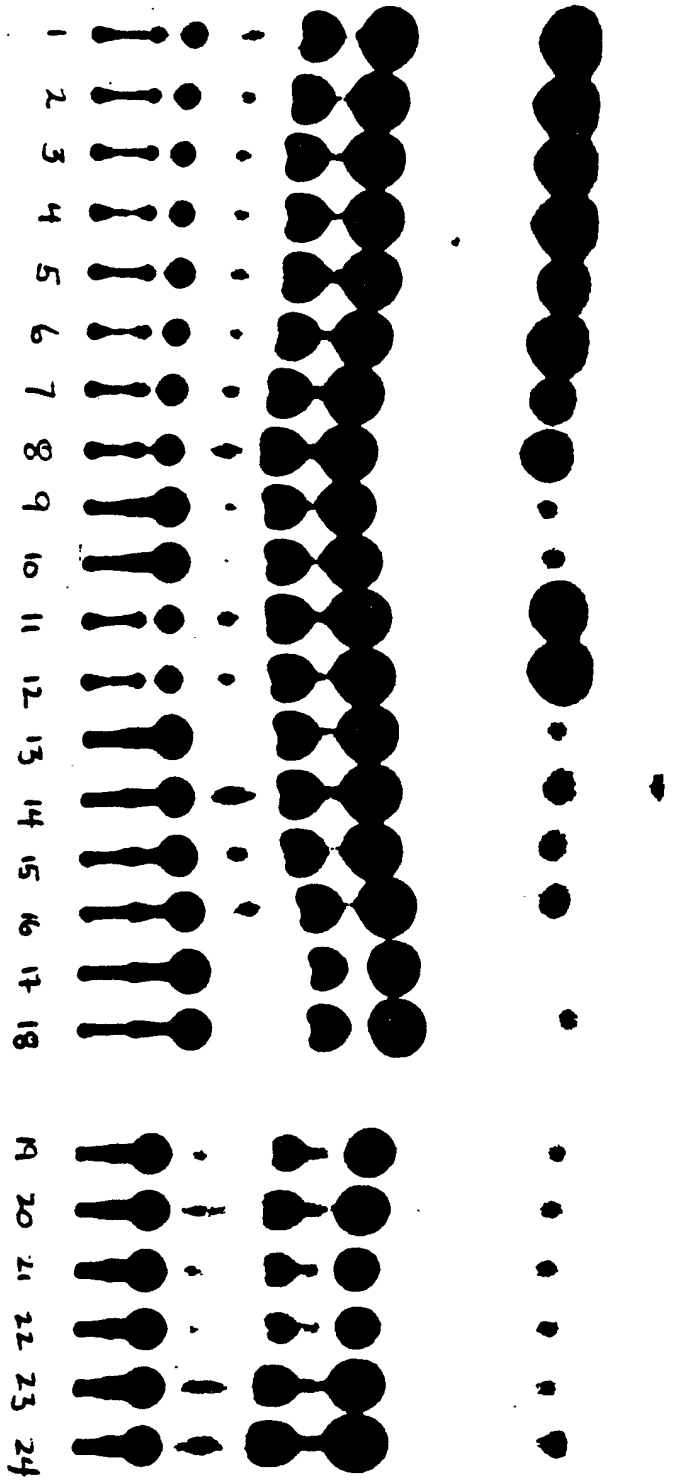


FIGURE 13

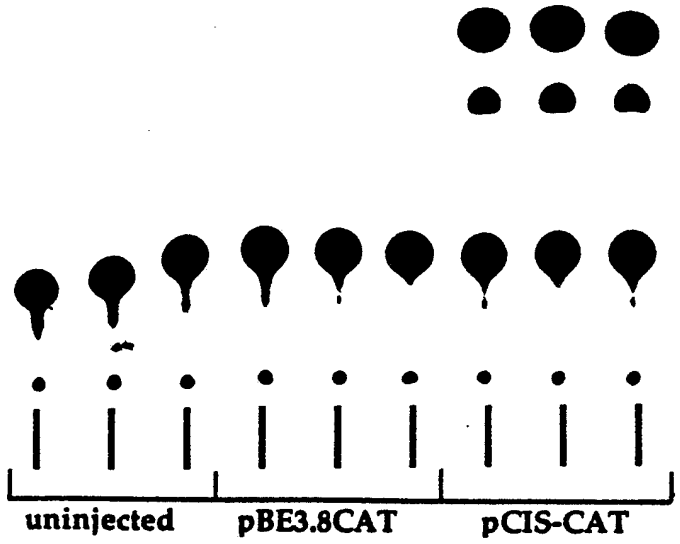


FIGURE 14A

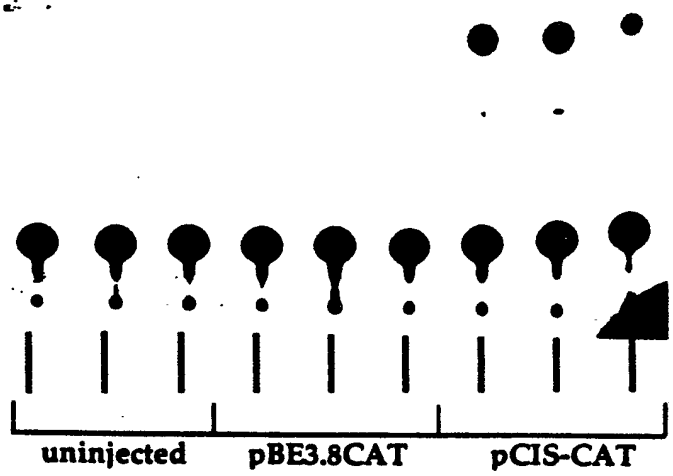


FIGURE 14B

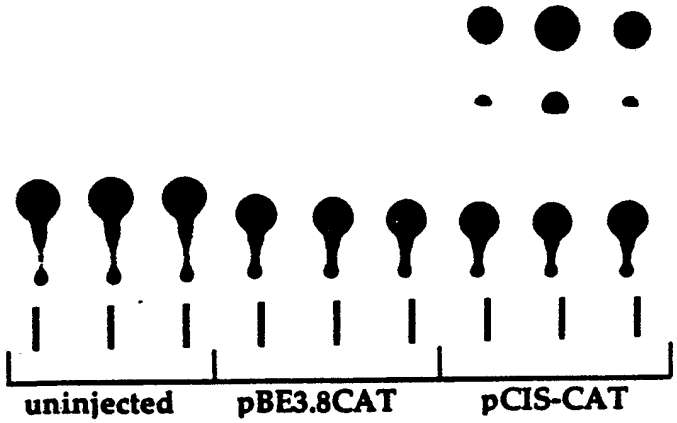


FIGURE 14C

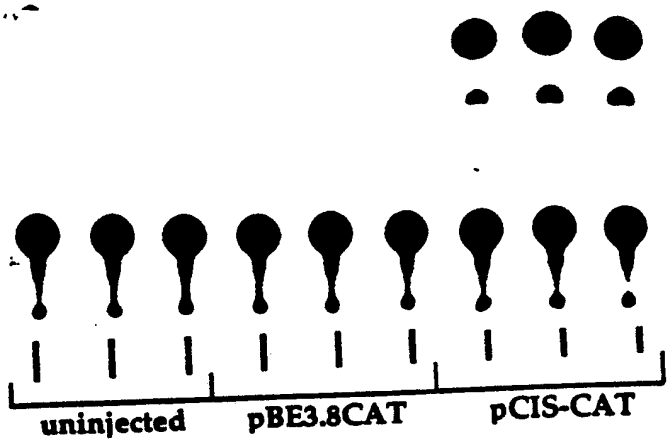


FIGURE 14D

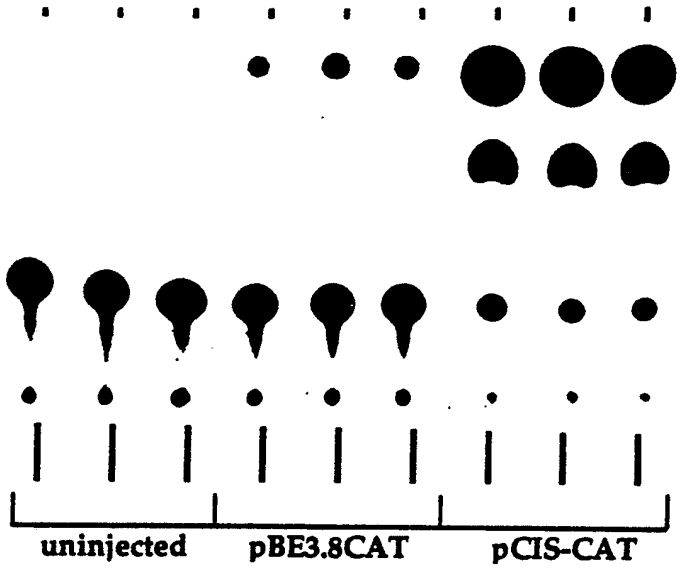


FIGURE 14E

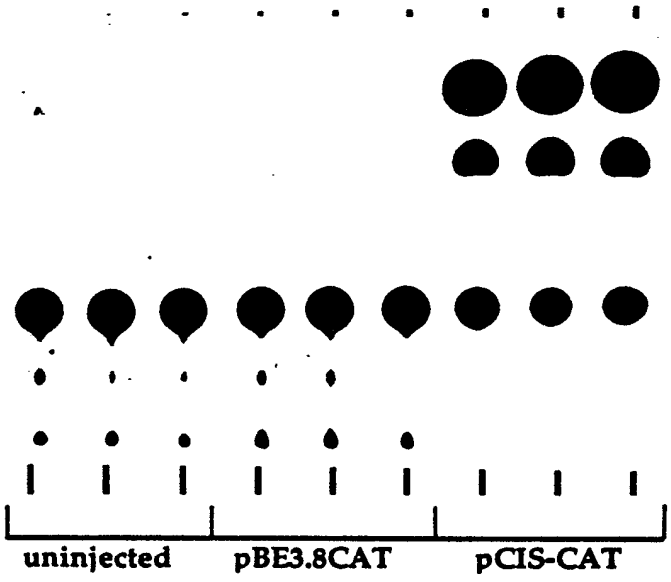


FIGURE 14F

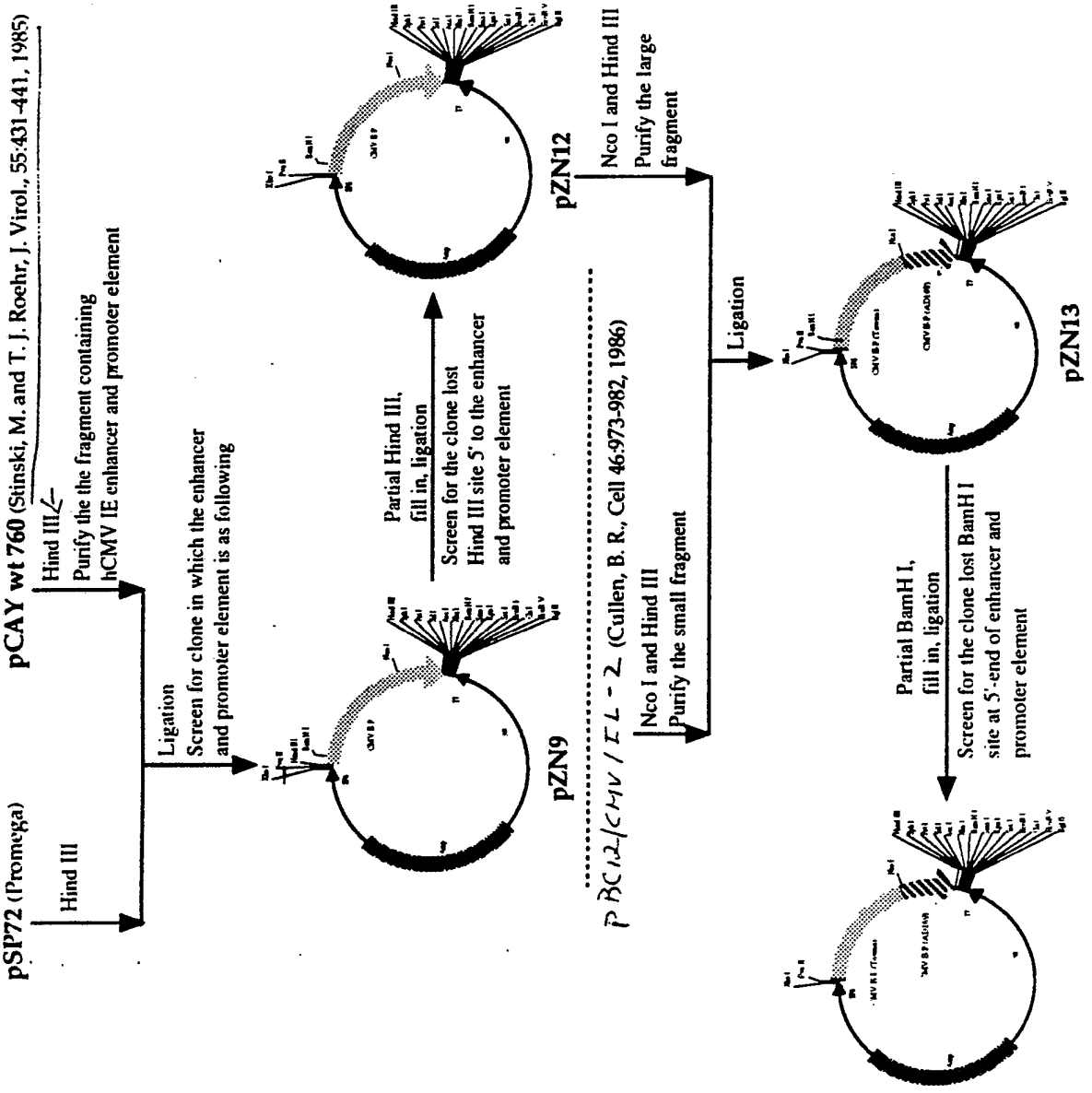

pCAY wt 760 (Stinski, M. and T. J. Roehr, J. Virol., 55:431-441, 1985)
Hind III
Purify the the fragment containing hCMV IE enhancer and promoter element
pSP72 (Promega)
Hind III
Ligation
Screen for clone in which the enhancer and promoter element is as following
pZN9
Partial Hind III, fill in, ligation
Screen for the clone lost Hind III site 5' to the enhancer and promoter element
pZN12
pBC12/CMV/IL-2 (Cullen, B. R., Cell 46:973-982, 1986)
Nco I and Hind III
Purify the small fragment
Nco I and Hind III
Purify the large fragment
Ligation
pZN13
Partial BamH I, fill in, ligation
Screen for the clone lost BamH I site at 5'-end of enhancer and promoter element

FIGURE 15

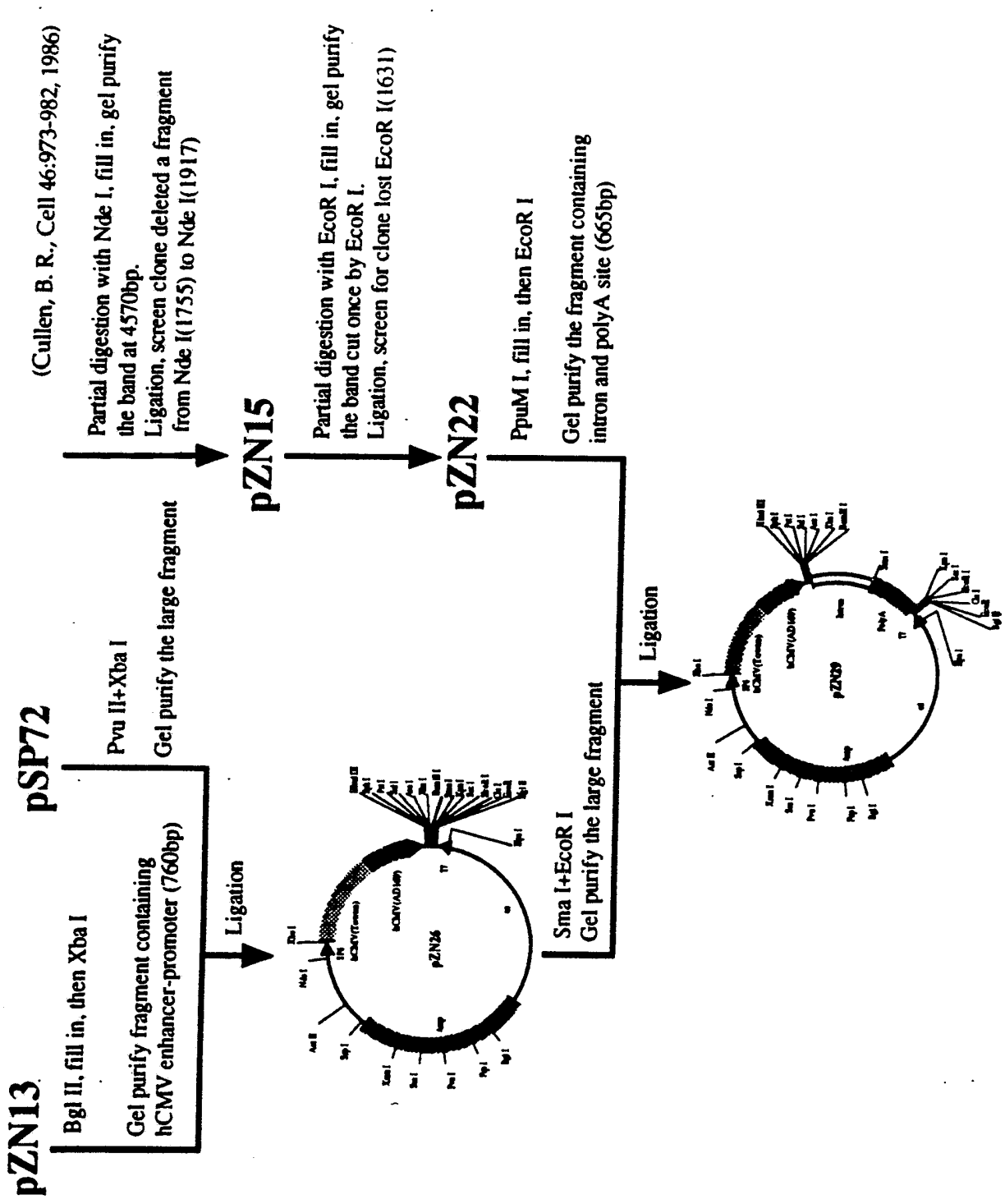

pZN13
Bgl II, fill in, then Xba I
Gel purify fragment containing hCMV enhancer-promoter (760bp)
pSP72
Pvu II+Xba I
Gel purify the large fragment
Ligation
(Cullen, B. R., Cell 46:973-982, 1986)
Partial digestion with Nde I, fill in, gel purify the band at 4570bp.
Ligation, screen clone deleted a fragment from Nde I(1755) to Nde I(1917)
pZN15
Partial digestion with EcoR I, fill in, gel purify the band cut once by EcoR I.
Ligation, screen for clone lost EcoR I(1631)
pZN22
PpuM I, fill in, then EcoR I
Gel purify the fragment containing intron and polyA site (665bp)
Sma I+EcoR I
Gel purify the large fragment
Ligation
pZN26
pZN29

FIGURE 16

pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)

Pst I, fill in, gel purify CFTR cDNA insert (~4.7kb)

pZN29

Sma I

Ligation
Screen for clone containing insert
in the sense orietation

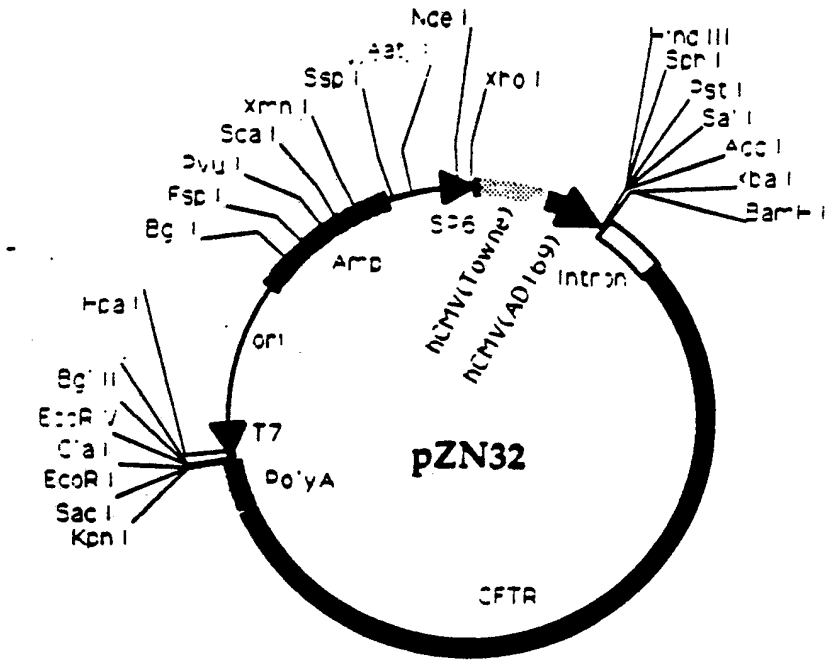


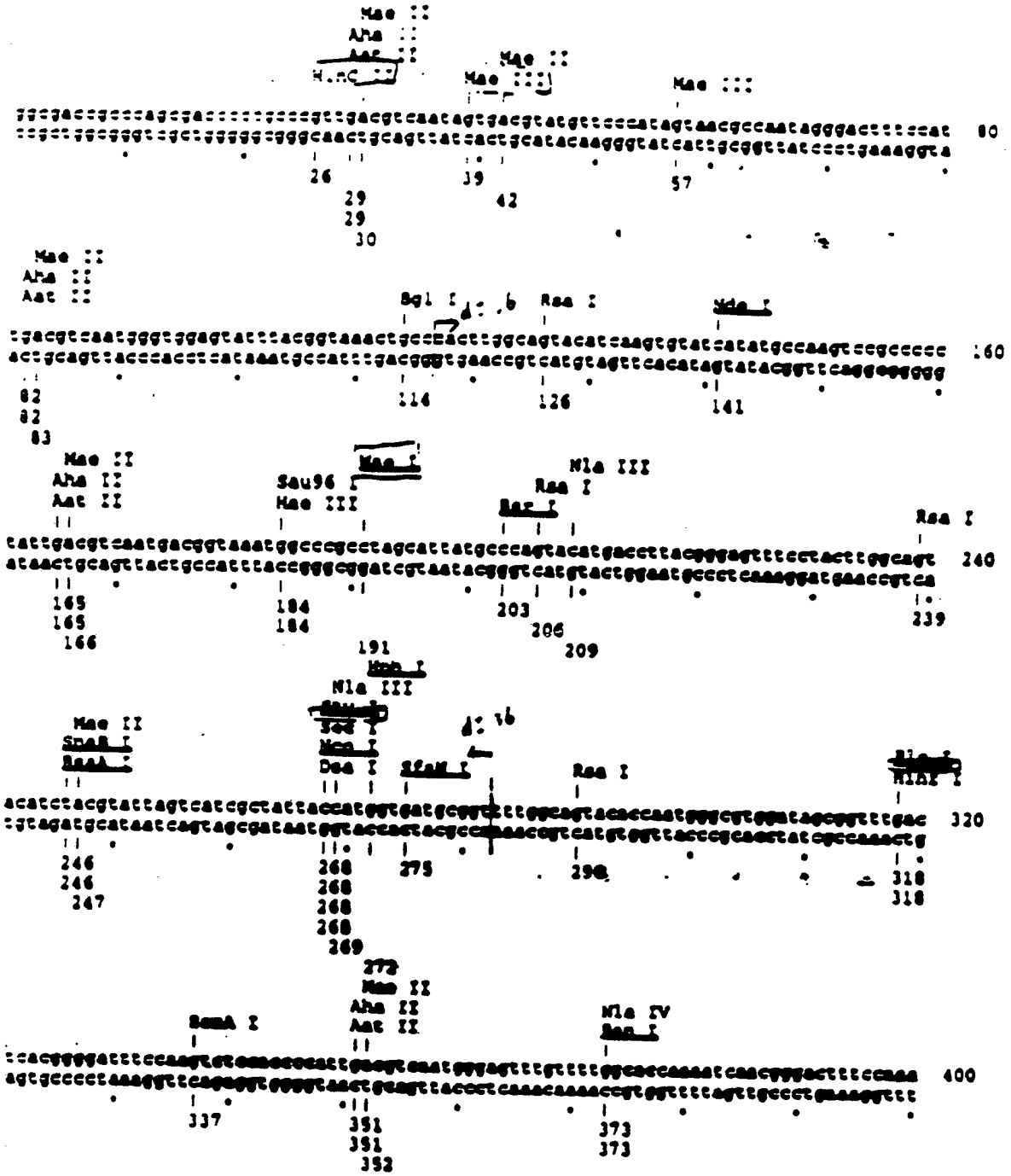
FIGURE 17

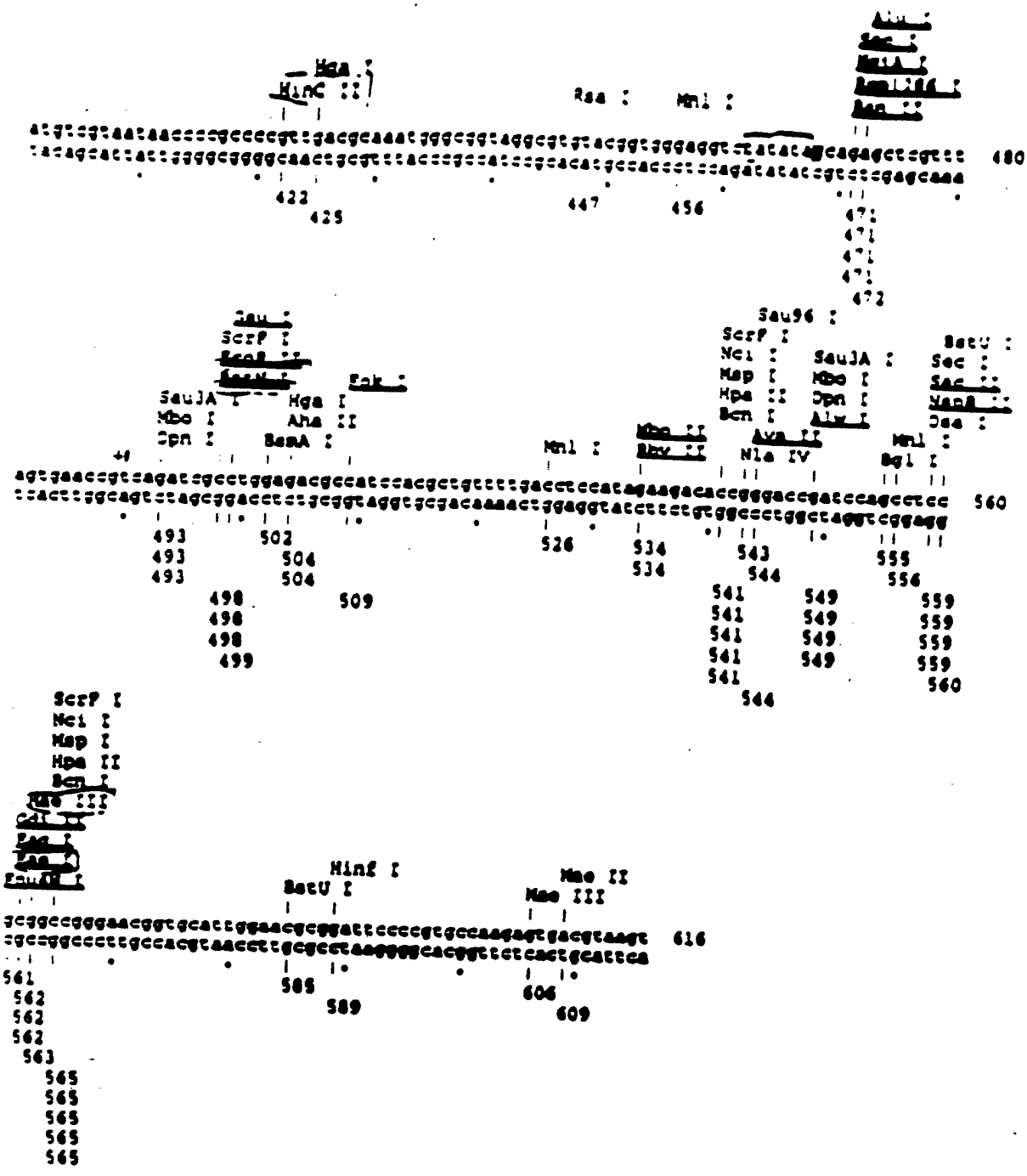
DNA Sorter™ 1.1 Tuesday, March 1, 1992

HCMV (Towne) -> Full Restriction Map

DNA sequence 616 b.p. ggcgaccgccca .. agtgacgtaagt linear

Positions of Restriction Endonucleases sites (unique sites underlined)



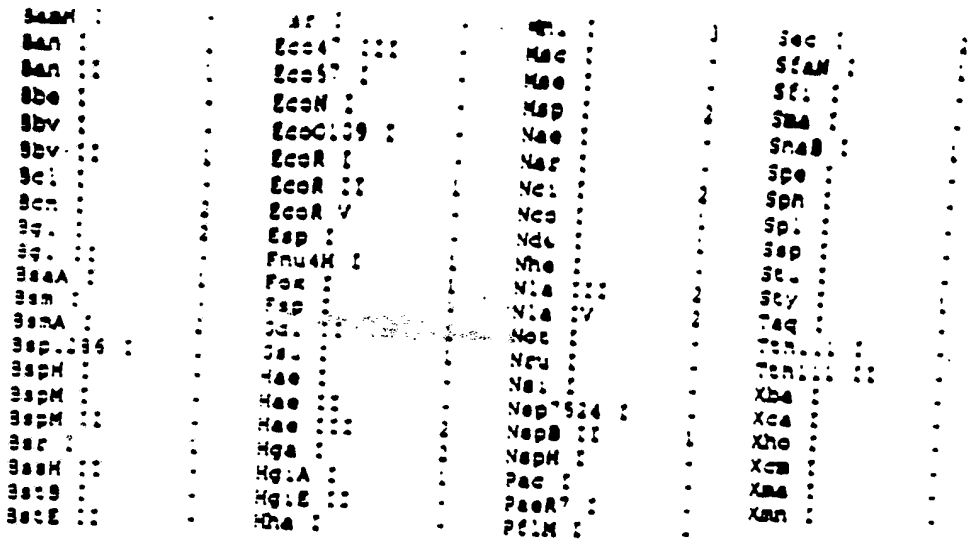


Restriction Endonucleases site usage

Aat II	4	BstN I	1	HinC II	2	Ple I	1
Acc I	-	BstU I	2	HinD III	-	Pml I	-
Afl II	-	BstX I	-	Hinf I	2	PpuM I	-
Afl III	-	BstY I	-	HinP I	-	Pst I	-
Aha II	5	Bsu36 I	-	Hpa I	-	Pvu I	-
Alu I	1	Cfr10 I	-	Hpa II	2	Pvu II	-
Alw I	1	Cla I	-	Hph I	1	Rsa I	5
AlwN I	-	Dde I	-	Kpn I	-	Rsr II	-
Apa I	-	Dpn I	2	Mae I	1	Sac I	1
ApaL I	-	Dra I	-	Mae II	7	Sac II	1
Ase I	-	Dra III	-	Mae III	3	Sal I	-
Asp718	-	Drd I	-	Mbo I	2	SauJA I	2
Ava I	-	Eae I	2	Mbo II	1	Sau96 I	2
Ava II	1	Eae I	1	Mlu I	-	Sca I	-
Avr II	-	Eag I	1	Mse I	-	ScrF I	3

FIGURE 19A(2)

HCMV Terminal > Full Restriction Map



Enzyme	Site	Use	Site position (Fragment length) Fragment order									
Alu I	ag/ct	1	1(471) 1	472(145) 2								
Alw I	ggatc 4/5	1	1(548) 1	549(68) 2								
Ava II	g/gwcc	1	1(543) 1	544(73) 2								
Ban I	g/gyrcc	1	1(372) 1	373(244) 2								
Ban II	grgcy/c	1	1(470) 1	471(146) 2								
Bbv II	gaagac 2/6	1	1(533) 1	534(83) 2								
BsaA I	yac/gtr	1	1(245) 2	246(371) 1								
Bsp1286 I	gdgch/c	1	1(470) 1	471(146) 2								
Bsr I	actgg 1/-1	1	1(202) 2	203(414) 1								
BstN I	cc/wgg	1	1(497) 1	498(119) 2								
Eae I	y/ggccr	1	1(561) 1	562(55) 2								
Eag I	c/ggccg	1	1(561) 1	562(55) 2								
EcoR II	/ccwgg	1	1(497) 1	498(119) 2								
Fnu4H I	gc/ngc	1	1(560) 1	561(56) 2								
Fok I	ggatg 9/13	1	1(508) 1	509(108) 2								
Gdi II	yggccg -5/-1	1	1(561) 1	562(55) 2								
Gsu I	ctggag 16/14	1	1(498) 1	499(118) 2								
Hga I	gwgcw/c	1	1(470) 1	471(146) 2								
Hph I	ggtga 8/7	1	1(271) 2	272(345) 1								
Mae I	c/tag	1	1(190) 2	191(426) 1								
Mbo II	gaaga 8/7	1	1(533) 1	534(83) 2								
Nco I	c/catgg	1	1(267) 2	268(349) 1								
Nde I	ca/tatg	1	1(140) 2	141(476) 1								
NspB II	cmg/ckg	1	1(558) 1	559(58) 2								
Ple I	gagtc 4/5	1	1(317) 1	318(299) 2								
Sac I	gagct/c	1	1(470) 1	471(146) 2								
Sac II	ccgc/gg	1	1(558) 1	559(58) 2								
SfaN I	gcatc 5/9	1	1(274) 2	275(342) 1								
SnaB I	tac/gta	1	1(245) 2	246(371) 1								
Sty I	c/cwwgg	1	1(267) 2	268(349) 1								
Ban I	ccs/gg	2	1(540) 1	541(24) 3	565(52) 2							
Bgl I	gccnnnn/nggc	2	1(113) 2	114(441) 1	555(62) 3							
BsmA I	gtctc 1/5	2	1(336) 1	337(165) 2	502(115) 3							
BstU I	cg/cg	2	1(559) 1	560(25) 3	585(32) 2							
Dpn I	ga/tc	2	1(492) 1	493(56) 3	549(68) 2							
Dsa I	c/crygg	2	1(267) 2	268(291) 1	559(58) 3							
Hae III	gg/cc	2	1(183) 2	184(379) 1	563(54) 3							
Hga I	gacgc 5/10	2	1(424) 1	425(79) 3	504(113) 2							
HinC II	gty/rac	2	1(25) 3	26(396) 1	422(195) 2							
Hinf I	g/antc	2	1(317) 1	318(271) 2	589(28) 3							
Hpa II	c/cgg	2	1(540) 1	541(24) 3	565(52) 2							
Mbo I	/gatc	2	1(492) 1	493(56) 3	549(68) 2							
Msp I	c/cgg	2	1(540) 1	541(24) 3	565(52) 2							
Nci I	cc/sgg	2	1(540) 1	541(24) 3	565(52) 2							
Nla III	catg/	2	1(208) 2	209(60) 3	269(348) 1							
Nla IV	ggn/ncc	2	1(372) 1	373(170) 2	543(74) 3							
SauJA I	gatc	2	1(492) 1	493(56) 3	549(68) 2							
Sau96 I	g/gncc	2	1(183) 2	184(360) 1	544(73) 3							
Sec I	c/cngg	2	1(267) 2	268(291) 1	559(58) 3							

FIGURE 19A(3)

HCMV [illegible] Restriction Map [illegible]

Mae III	gtnac	3	38 2	391	181 3	57	549	5?6		
Mnl I	cctc	3	455	456	70 2	526	30 4	556		
ScrF I	cc/ngg	3	497	498	43 3	541	24 4	565		
Aat II	gacgt/c	4	29 5 / 351 266	29	53 4	82	83 3	165	136	
Aha II	gr/cgyc	5	28 6 / 351 153 2	29	53 5 / 524 [illegible] 3	82	83 4	165	136	
Fsa I	gt/ac	5	125 3 / 293 157 2	126	80 4 / 447 [illegible]	206	33 6	239	[illegible]	
Mae II	a/cgt	[illegible]	29 6 / 166 81 4	30	12 7 / 247 105 2	42	41 5 / 352 257	83 / 619	[illegible]	

[illegible] sites found

No Sites found for the following Restriction Endonucleases

Acc I	gt/mkac		Dra III	cacnnn/gtg		Nsp7524 I	r/catgy	
Afl II	c/ttaag		Drd I	gacnnnn/nngtc		NspH I	rcatg/y	
Afl III	a/crygt		Ear I	ctcttc	1/4	Pac I	ttaat/taa	
AlwN I	cagnnn/ctg		Eco47 III	agc/gct		PaeR7 I	c/tcgag	
Apa I	gggcc/c		Eco57 I	ctgaag	16/14	PflM I	ccannnn/ntgg	
ApaL I	g/tgcac		EcoN I	cctnn/nnnagg		Pml I	cac/gtg	
Ase I	at/taat		EcoO109 I	rg/gnccy		PpuM I	rg/gwccy	
Asp718	g/gtacc		EcoR I	g/aattc		Pst I	ctgca/g	
Ava I	c/ycgrg		EcoR V	gat/atc		Pvu I	cgat/cg	
Avr II	c/ctagg		Esp I	gc/tnagc		Pvu II	cag/ctg	
BamH I	g/gatcc		Fsp I	tgc/gca		Rsr II	cg/gwccg	
Bbe I	ggcgc/c		Hae I	wgg/ccw		Sal I	g/tcgac	
Bbv I	gcagc	8/12	Hae II	rgcgc/y		Sca I	agt/act	
Bcl I	t/gatca		HgiE II	accnnnnnnggt		Sfi I	ggccnnnn/nggcc	
Bgl II	a/gatct		Hha I	gcg/c		Sma I	ccc/ggg	
Bsm I	gaatgc	1/-1	HinD III	a/agctt		Spe I	a/ctagt	
BspH I	t/catga		HinP I	g/cgc		Sph I	gcatg/c	
BspM I	acctgc	4/8	Hpa I	gtt/aac		Spl I	c/gtacg	
BspM II	t/ccgga		Kpn I	ggtac/c		Ssp I	aat/att	
BssH II	g/cgcgc		Mlu I	a/cgcgt		Stu I	agg/cct	
BstB I	tt/cgaa		Mme I	tccrac	20/18	Taq I	t/cga	
BstE II	g/gtnacc		Msc I	tgg/cca		Tth111 I	gacn/nngtc	
BstX I	ccannnnn/ntgg		Mse I	t/taa		Tth111 II	caarca	11/9
BstY I	r/gatcy		Nae I	gcc/ggc		Xba I	t/ctaga	
Bsu36 I	cc/tnagg		Nar I	gg/cgcc		Xca I	gta/tac	
Cfr10 I	r/ccggy		Nhe I	g/ctagc		Xho I	c/tcgag	
Cla I	at/cgat		Not I	gc/ggccgc		Xcm I	ccannnnn/nnnntgg	
Dde I	c/tnag		Nru I	tcg/cga		Xma I	c/ccggg	
Dra I	ttt/aaa		Nsi I	atgca/t		Xmn I	gaann/nnttc	

FIGURE 19A(4)

```
*** Aligned sequences:
C1 ( 1f): |>u 1>------ ad169hcmv  930 bases|------>u 930>|
C2 ( 1f): |>u 1>------ hs5mie1  (616 bases)|------>u 616>|

*** Alignment of first sequence with all others displayed
*** Key:
    UPPER CASE = aligned non-identical bases
    lower case = unaligned bases
    ---------- = aligned identical bases
    .......... = gap

ad169hcmv : AATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGC
  hs5mie1 : ..........................................................

ad169hcmv : TATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGT
  hs5mie1 : ..........................................................

ad169hcmv : CCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACG
  hs5mie1 : ..........................................................

ad169hcmv : GGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
  hs5mie1 : ..........................................................

ad169hcmv : CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCC
  hs5mie1 : .....---.---------G----------G----------G----------

ad169hcmv : ATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACT
  hs5mie1 : ----------------------------------------------------------

ad169hcmv : GCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAAT
  hs5mie1 : ----------------------------------------C-----------------

ad169hcmv : GACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACT
  hs5mie1 : ------------------A-----------------------C----G---------

                                                    *
ad169hcmv : TGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTAC
  hs5mie1 : ----------------------------------------------------------

ad169hcmv : ATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC
  hs5mie1 : -C--------------------------------------------------------

ad169hcmv : GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAAC
  hs5mie1 : ----------------------------------------------------T---

ad169hcmv : TCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGA
  hs5mie1 : C-------G-------------------------------------------------

ad169hcmv : GCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCAT
  hs5mie1 : ----------------------------------------------------------

ad169hcmv : AGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATT
  hs5mie1 : ----------------------------------------------------------

ad169hcmv : CCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCT
  hs5mie1 : ----------------------.....................................

ad169hcmv : TCTTATGCATGCTATACTGTTTTTGGCTTG
  hs5mie1 : ...............................
```

FIGURE 19B(1)

```
LOCUS       HSIE2E          930 bp ds-DNA              VRL       15-SEP-1989
DEFINITION  Human cytomegalovirus major immediate-early gene, enhancer.
ACCESSION   K03104
KEYWORDS    major immediate-early gene.
SOURCE      HCMV strain AD169.
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1  bases 1 to 930)
  AUTHORS   Boshart,M., Weber,F., Jahn,G., Dorsch-Haesler,K., Fleckenstein,
            and Schaffner,W.
  TITLE     A very strong enhancer is located upstream of an immediate earl
            gene of human cytomegalovirus
  JOURNAL   Cell 41, 521-530 (1985)
  STANDARD  full staff_review
REFERENCE   2  (sites)
  AUTHORS   Zhang,X.-Y., Inamdar,N.M., Supakar,P.C., Wu,K., Ehrlich,M. and
            Ehrlich,K.C.
  TITLE     three MDBP sites in the immediate-early enhancer-promoter regio
            human cytomegalovirus
  JOURNAL   Virology 182, 865-869 (1991)
  STANDARD  full staff_review
COMMENT     Draft entry and printed copy of sequence in [1] were kindly
            provided by M.Boshart, 24-OCT-1985.
FEATURES             Location/Qualifiers
     misc_signal     214..620
                     /note="HCMV IE enhancer region"
     mRNA            738..>930
                     /note="HCMV IE mRNA"
BASE COUNT      233 a    228 c    211 g    258 t
ORIGIN      12 bp upstream of BalI site; .750 mu.
        1 aatcaatatt ggccattagc catattattc attggttata tagcataaat caatattggc
       61 tattggccat tgcatacgtt gtatccatat cataatatgt acatttatat tggctcatgt
      121 ccaacattac cgccatgttg acattgatta ttgactagtt attaatagta atcaattacg
      181 gggtcattag ttcatagccc atatatggag ttccgcgtta cataacttac ggtaaatggc
      241 ccgcctggct gaccgcccaa cgacccccgc ccattgacgt caataatgac gtatgttccc
      301 atagtaacgc caatagggac tttccattga cgtcaatggg tggagtattt acggtaaact
      361 gcccacttgg cagtacatca agtgtatcat atgccaagta cgcccccctat tgacgtcaat
      421 gacggtaaat ggcccgcctg gcattatgcc cagtacatga ccttatggga ctttcctact
      481 tggcagtaca tctacgtatt agtcatcgct attaccatgg tgatgcggtt ttggcagtac
      541 atcaatgggc gtggatagcg gtttgactca cggggatttc caagtctcca ccccattgac
      601 gtcaatggga gtttgttttg gcaccaaaat caacgggact ttccaaaatg tcgtaacaac
      661 tccgccccat tgacgcaaat gggcggtagg cgtgtacggt gggaggtcta tataagcaga
      721 gctcgtttag tgaaccgtca gatcgcctgg agacgccatc cacgctgttt tgacctccat
      781 agaagacacc gggaccgatc cagcctccgc ggccgggaac ggtgcattgg aacgcggatt
      841 ccccgtgcca agagtgacgt aagtaccgcc tatagagtct ataggcccac ccccttggct
      901 tcttatgcat gctatactgt ttttggcttg
```

FIGURE 19B(2)

```
LOCUS       HSSMIE1        616 bp ds-DNA             VRL       15-SEP-1989
DEFINITION  Human cytomegalovirus (Towne) major immediate-early (IE) gene, excn
            1.
ACCESSION   K01484 K01090
KEYWORDS    major immediate-early gene.
SOURCE      Human cytomegalovirus (strain Towne) passed in primary human
            foreskin fibroblasts, DNA [1], clone pXEP22 [2].
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1  (bases 460 to 616)
  AUTHORS   Stenberg,R.M., Thomsen,D.R. and Stinski,M.F.
  TITLE     Structural analysis of the major immediate early gene of human
            cytomegalovirus
  JOURNAL   J. Virol. 49, 190-199 (1984)
  STANDARD  full staff_review
REFERENCE   2  (bases 1 to 490)

Enable your screen-to-printer option; tap RETURN
EuGene Project: CMV report: (GenBank entry information)
LOCUS       HSSMIE1        616 bp ds-DNA             VRL       15-SEP-1989
DEFINITION  Human cytomegalovirus (Towne) major immediate-early (IE) gene, excn
            1.
ACCESSION   K01484 K01090
KEYWORDS    major immediate-early gene.
SOURCE      Human cytomegalovirus (strain Towne) passed in primary human
            foreskin fibroblasts, DNA [1], clone pXEP22 [2].
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1  (bases 460 to 616)
  AUTHORS   Stenberg,R.M., Thomsen,D.R. and Stinski,M.F.
  TITLE     Structural analysis of the major immediate early gene of human
            cytomegalovirus
  JOURNAL   J. Virol. 49, 190-199 (1984)
  STANDARD  full staff_review
REFERENCE   2  (bases 1 to 490)
  AUTHORS   Thomsen,D.R., Stenberg,R.M., Goins,W.F. and Stinski,M.F.
  TITLE     Promoter-regulatory region of the major immediate early gene of
            human cytomegalovirus
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 81, 659-663 (1984)
  STANDARD  full staff_review
COMMENT     IE region 1 gene is also known as the major IE gene.
FEATURES             Location/Qualifiers
     prim_transcript 490..>616
                     /note="major IE mRNA"
     intron          611..>616
                     /note="major IE mRNA intron A"
BASE COUNT      144 a    165 c    162 g    145 t
ORIGIN      28 bp upstream of HincII site; 0.752 map units.
        1 ggcgaccgcc cagcgaccca cgcccgttga cgtcaatagt gacgtatgtt cccatagtaa
       61 cgccaatagg gactttccat tgacgtcaat gggtgggagta tttacggtaa actgcccact
      121 tggcagtaca tcaagtgtat catatgccaa gtccgccccc tattgacgtc aatgacggta
      181 aatggcccgc ctagcattat gcccagtaca tgaccttacg gggagtttcct acttggcagt
      241 acatctacgt attagtcatc gctattacca tggtgatgcg gttttggcag tacaccaatg
      301 ggcgtggata gcggtttgac tcacggggat ttccaagtct ccaccccatt gacgtcaatg
      361 ggagtttgtt ttggcaccaa aatcaacggg actttccaaa atgtcgtaat aaccccgccc
      421 cgttgacgca aatgggcggt aggcgtgtac ggtgggaggt ctatataagca gagctcgttt
      481 agtgaaccgt cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca
      541 ccgggaccga tccagcctcc gcggccgggg acggtgcatt ggaacgcgga ttccccgtgc
      601 caagagtgac gtaagt
```

FIGURE 19B(3)

*** DNA Strider™ 1 *** Wednesday, July 1, 1992 5:44:51 PM

HCMV (AD169) -> Restriction Map

DNA sequence 930 b.p. aattaatattgg .. gttttttggtttg ..naar

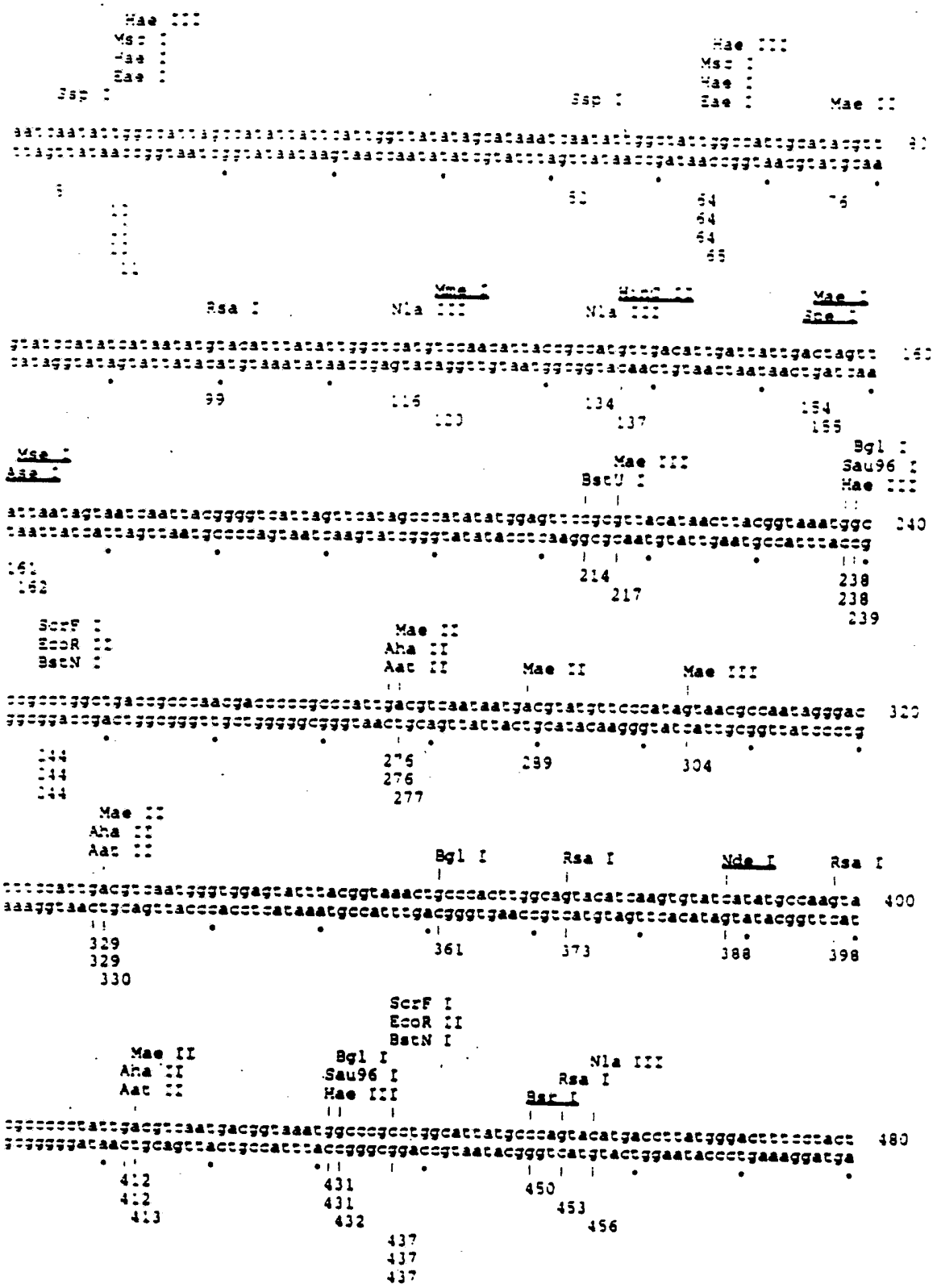


FIGURE 19C(1)

HCMV AD169 -> Restriction Map

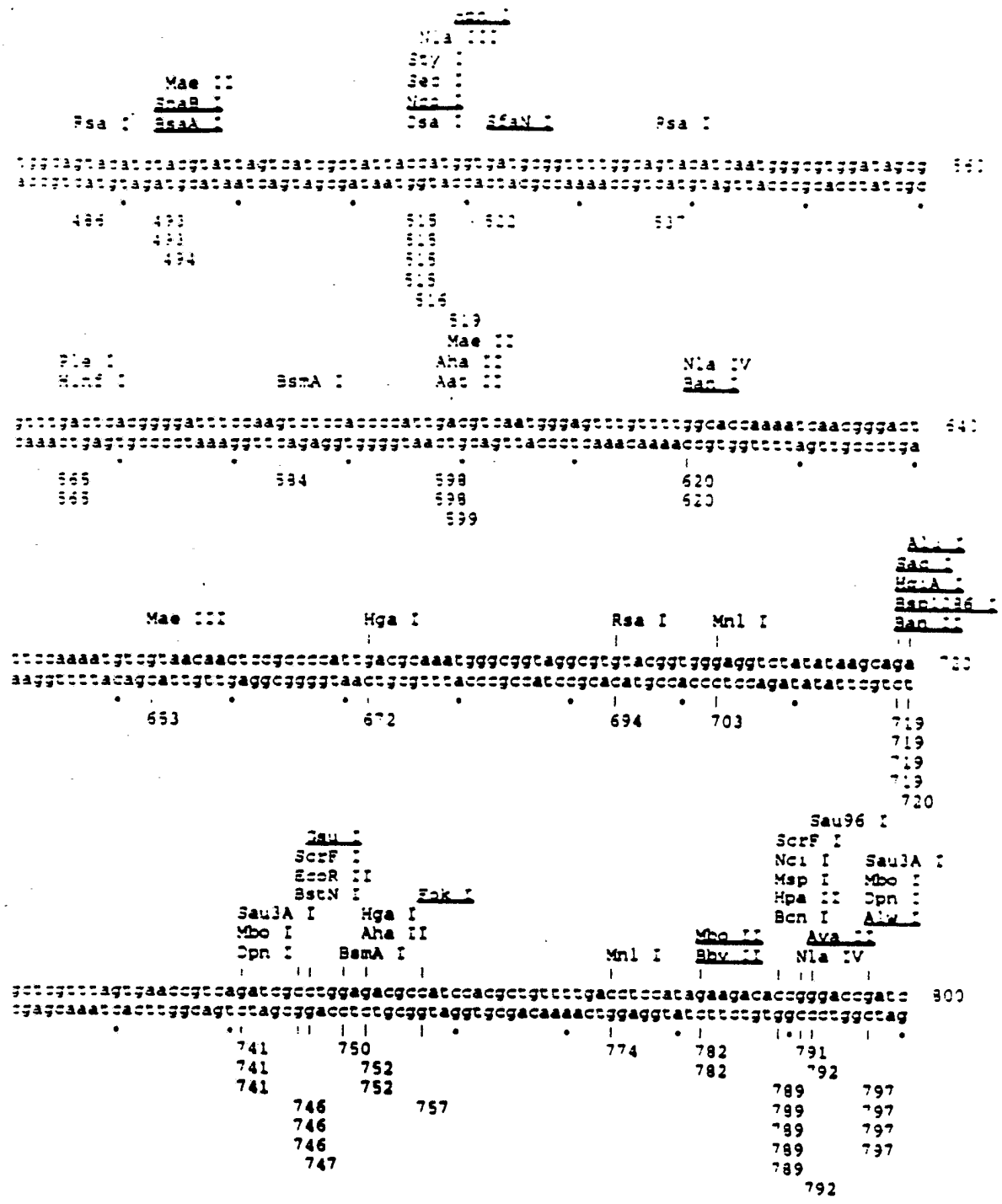


FIGURE 19C(2)

HCMV AD169 -> Restriction Map

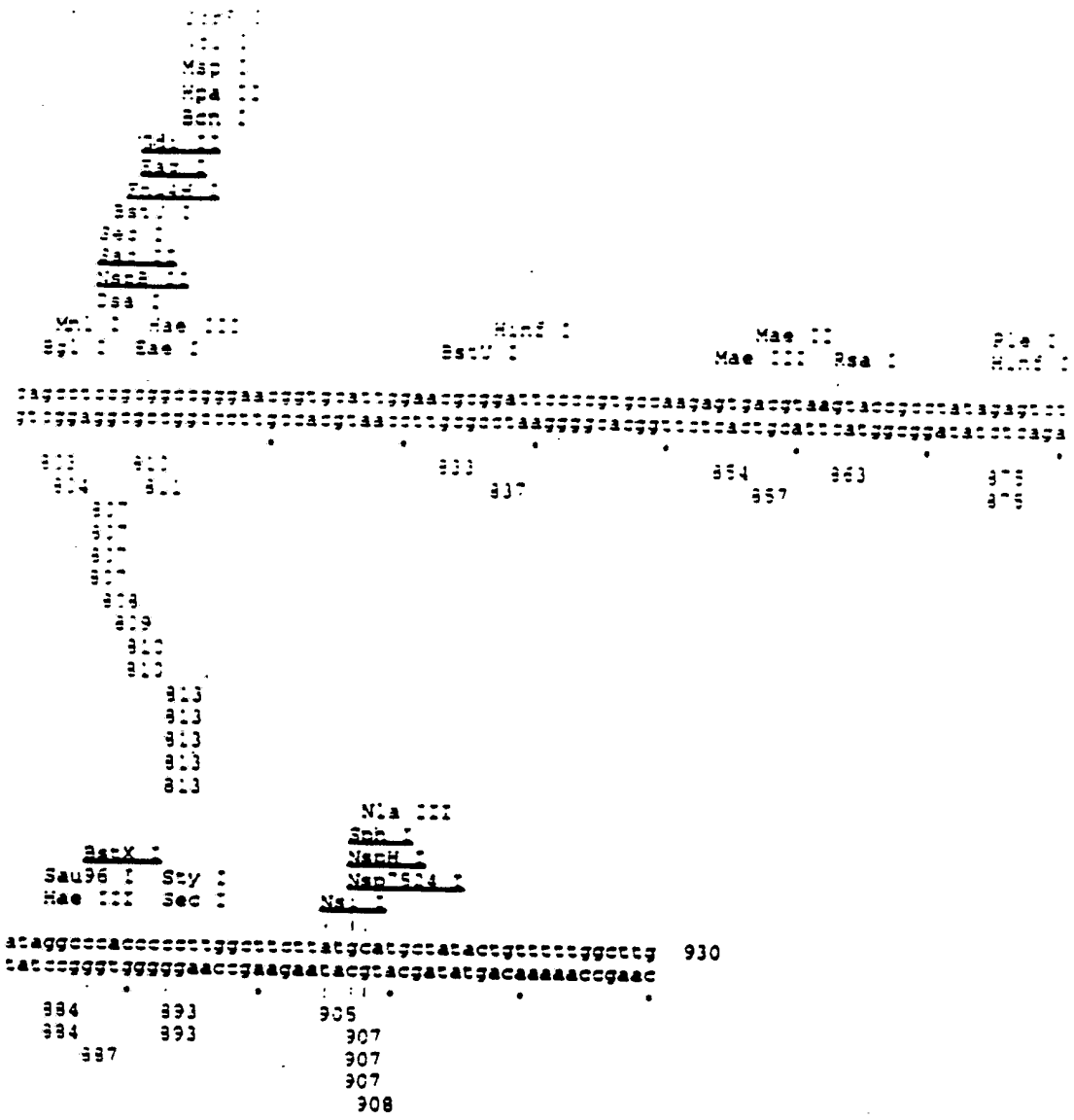


FIGURE 19C(3)

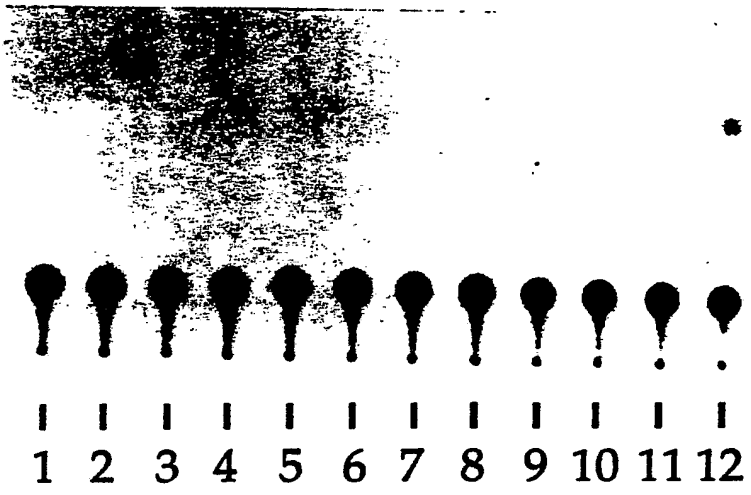


FIGURE 20

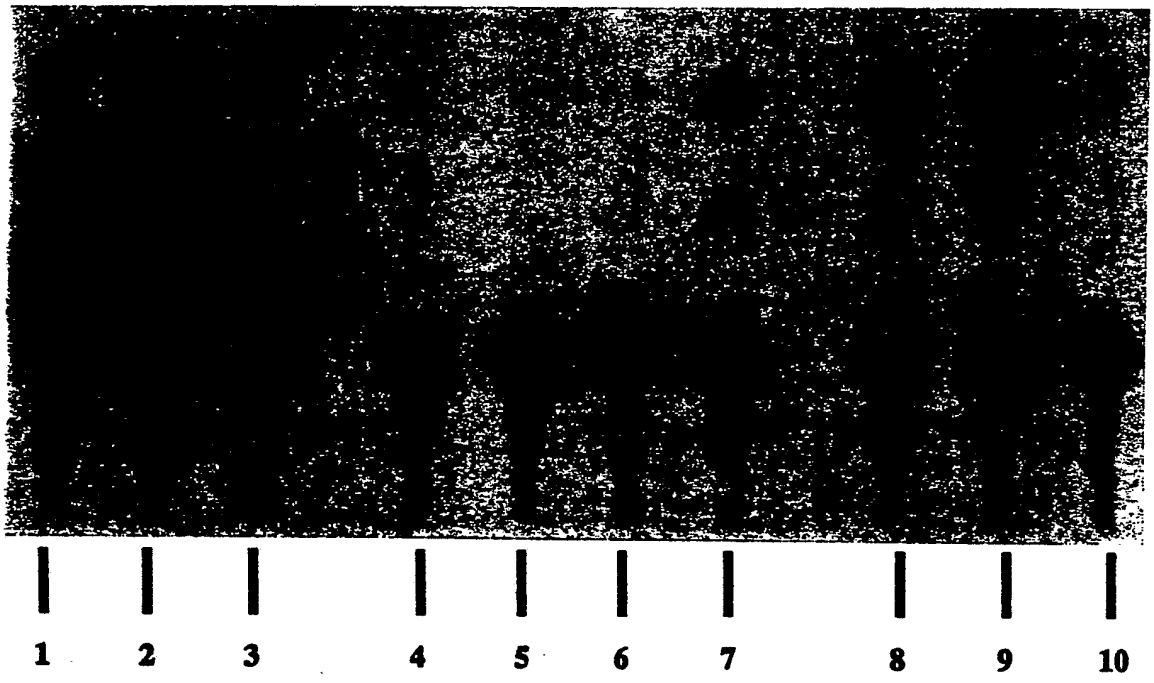


FIGURE 21

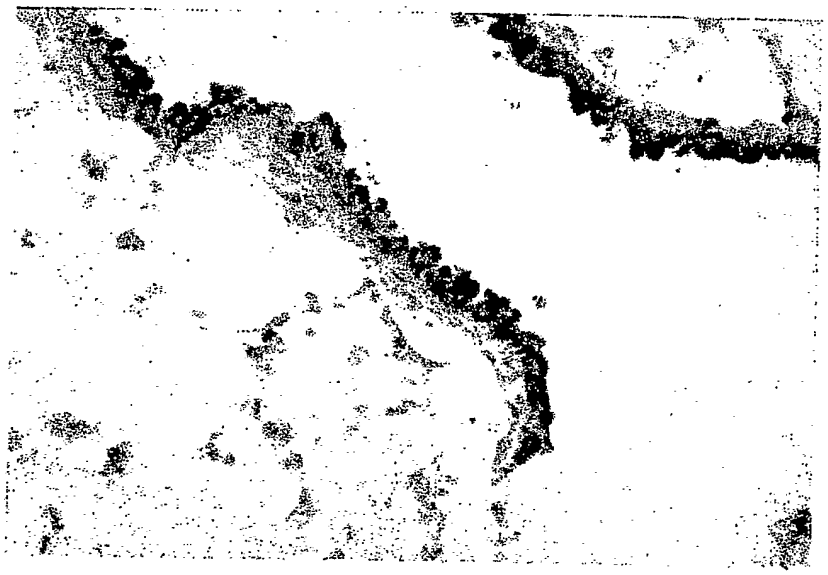


FIGURE 22A

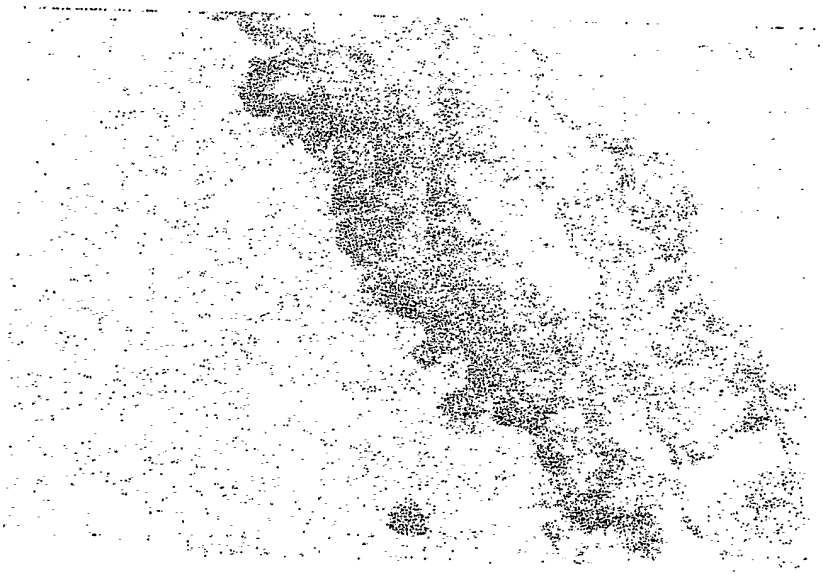


FIGURE 22B

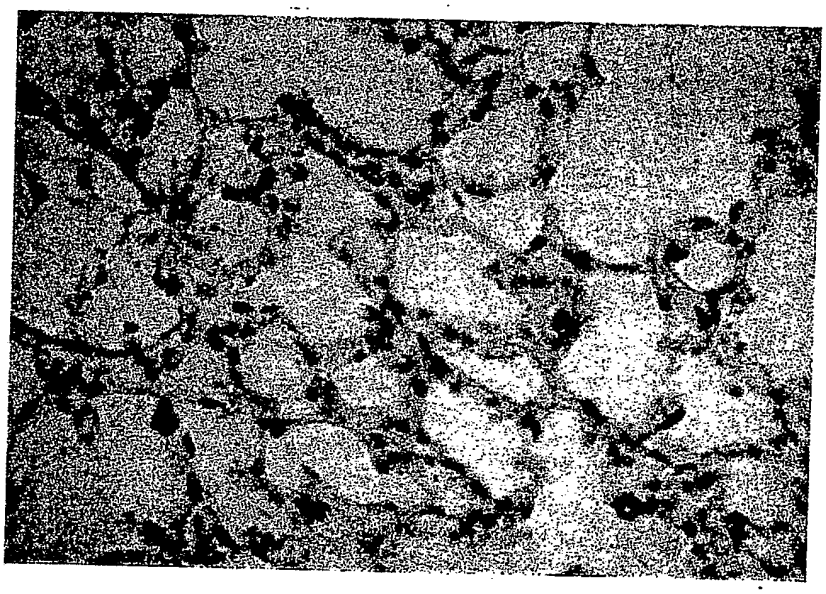


FIGURE 22C

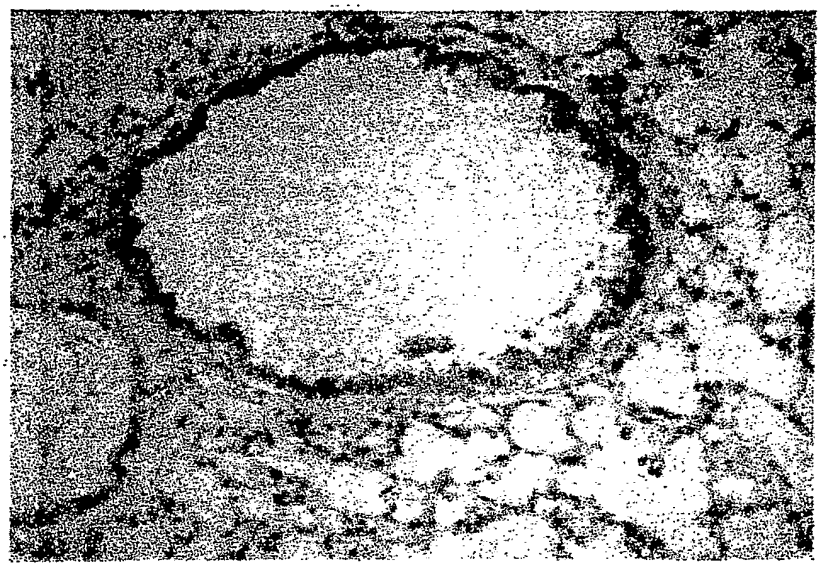


FIGURE 22D

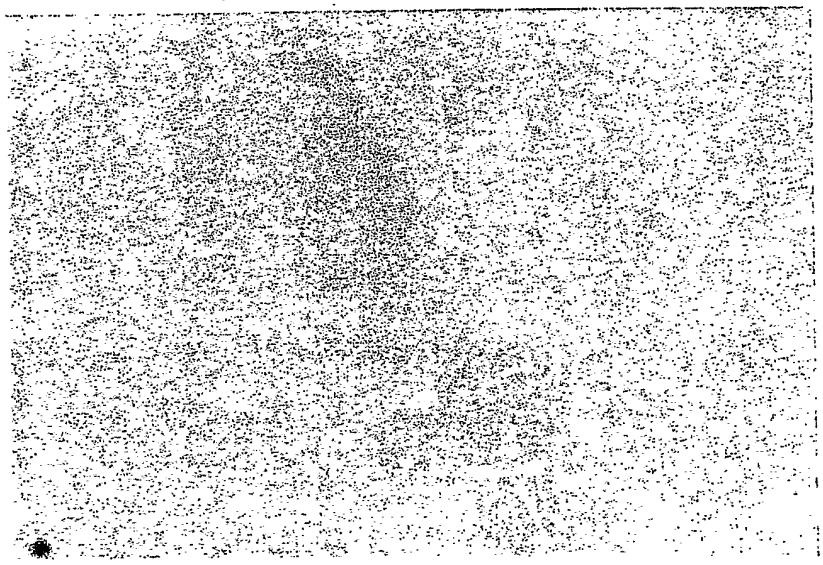


FIGURE 22E

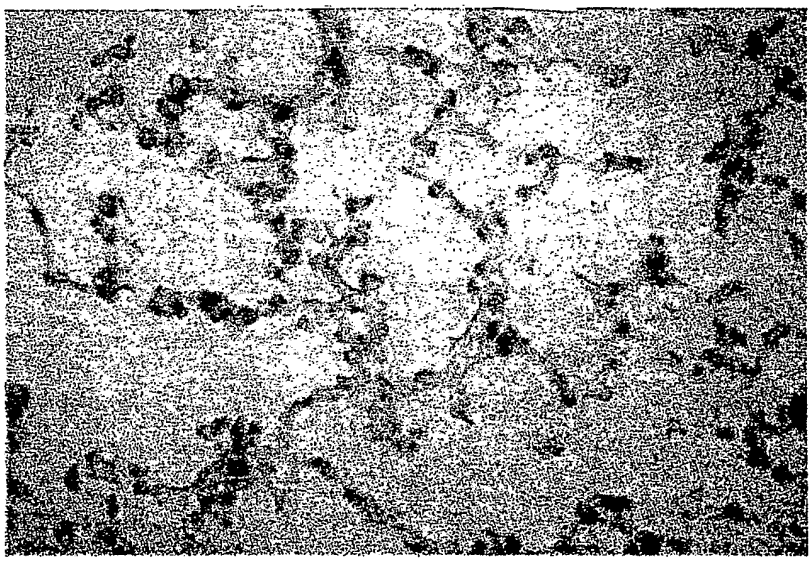


FIGURE 22F

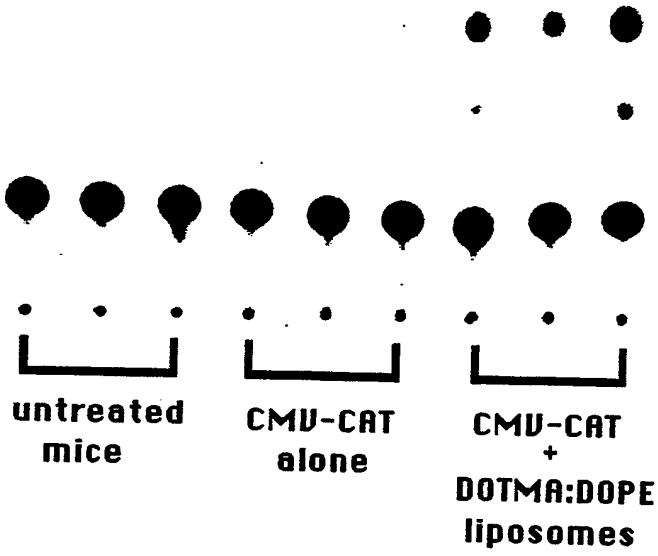


FIGURE 23

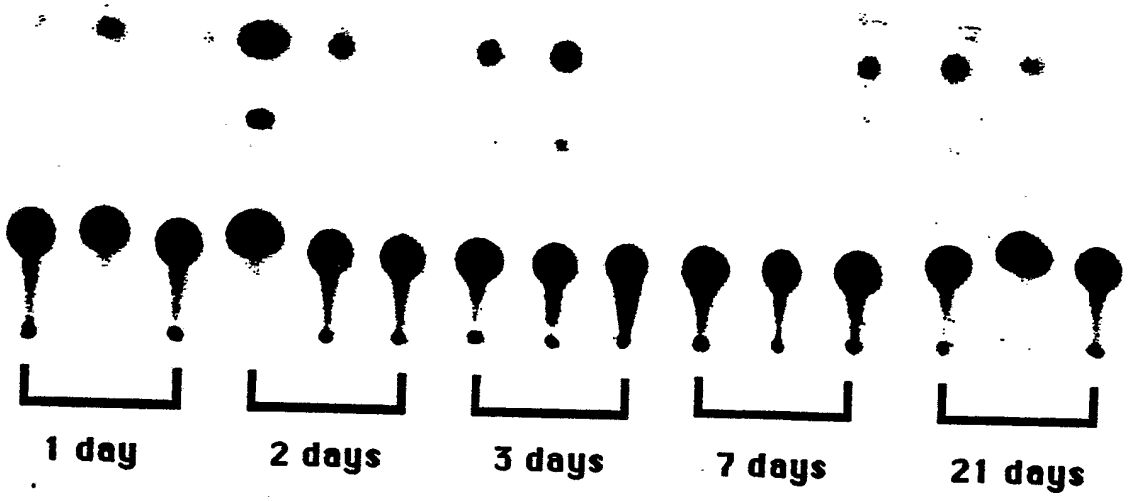


FIGURE 24A

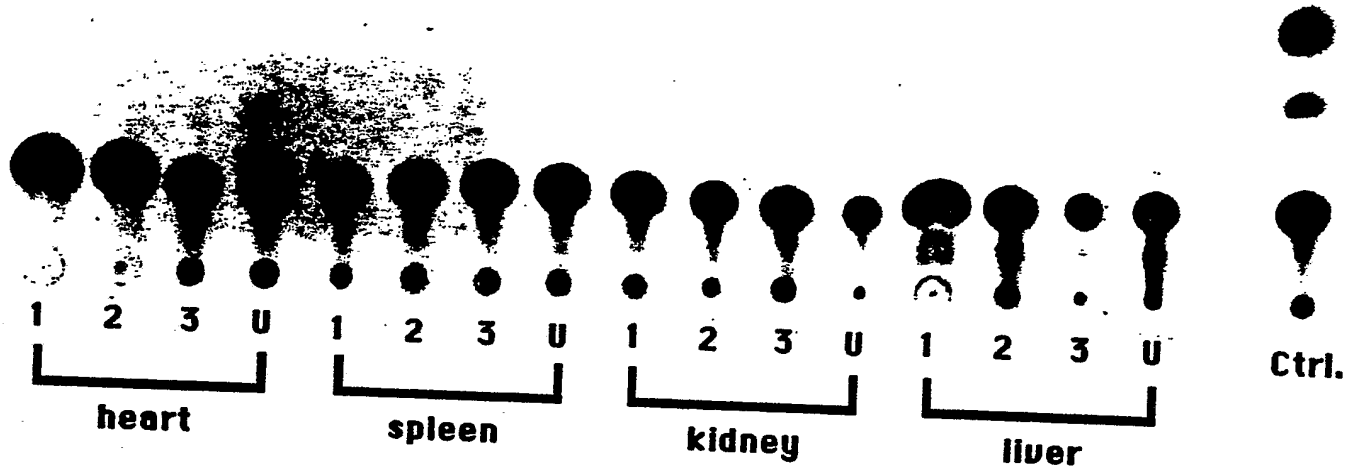


FIGURE 24B

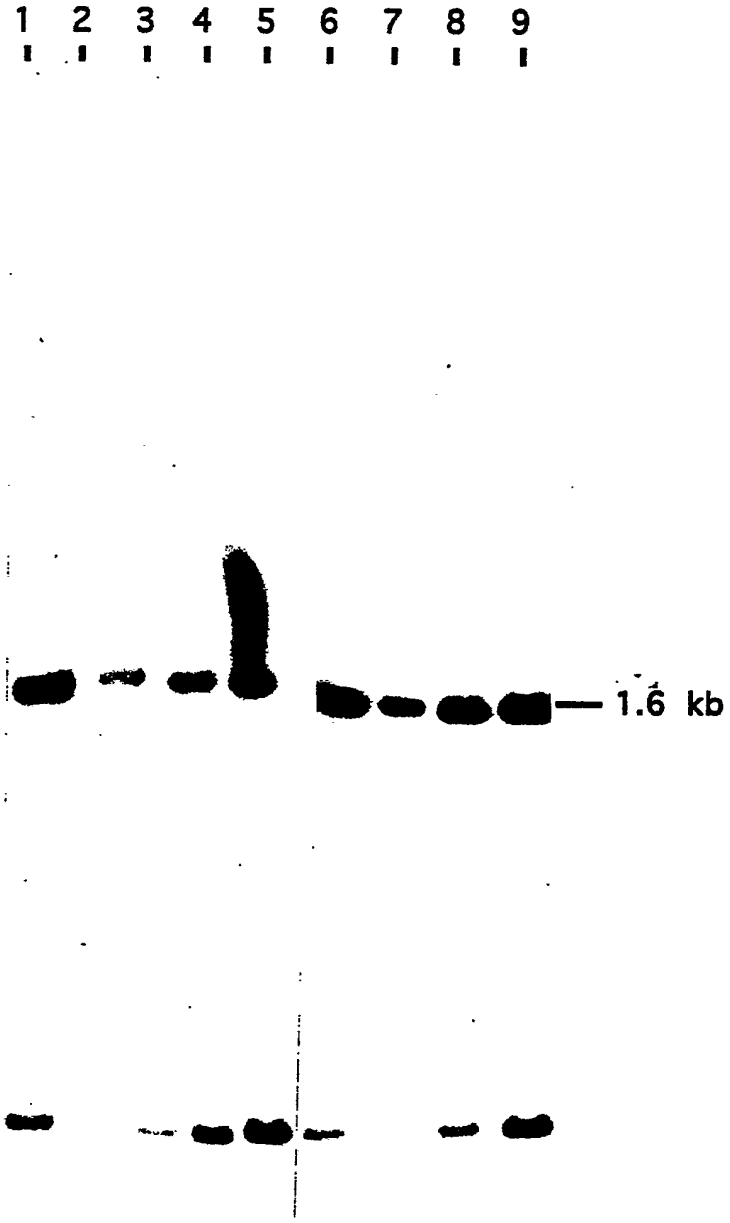


FIGURE 25

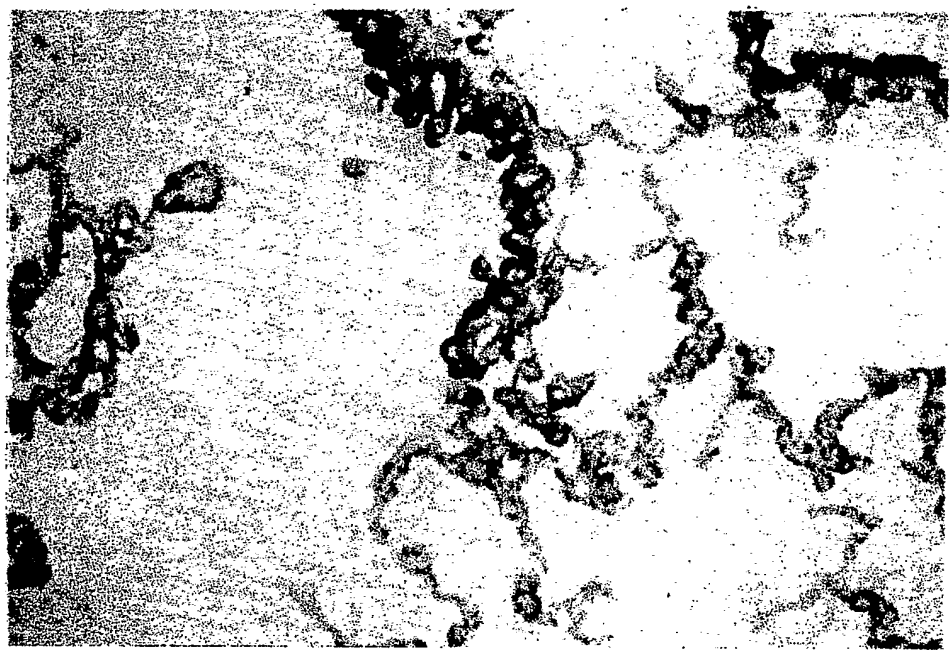


FIGURE 26A

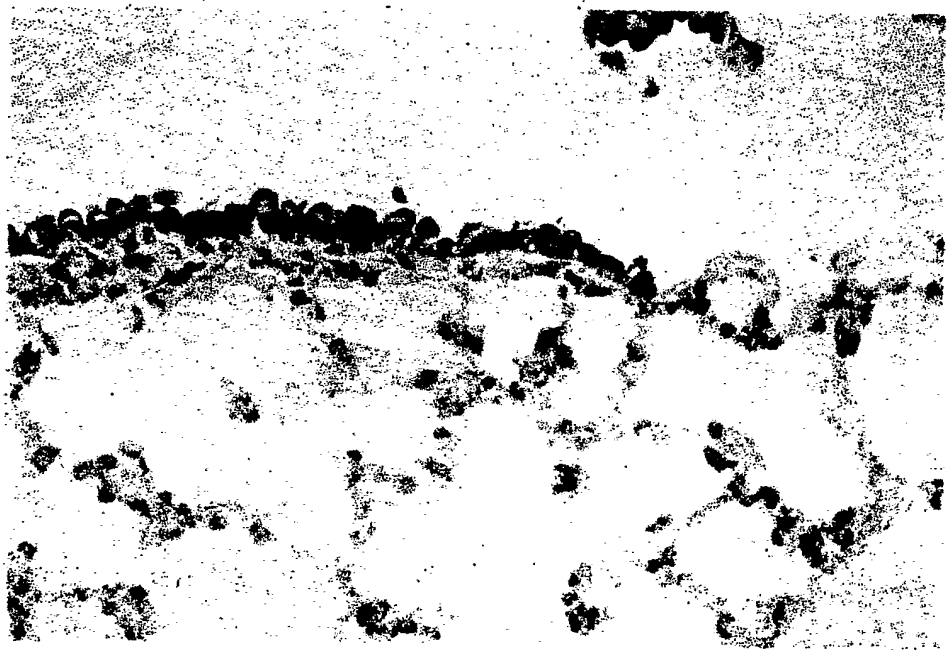


FIGURE 26B

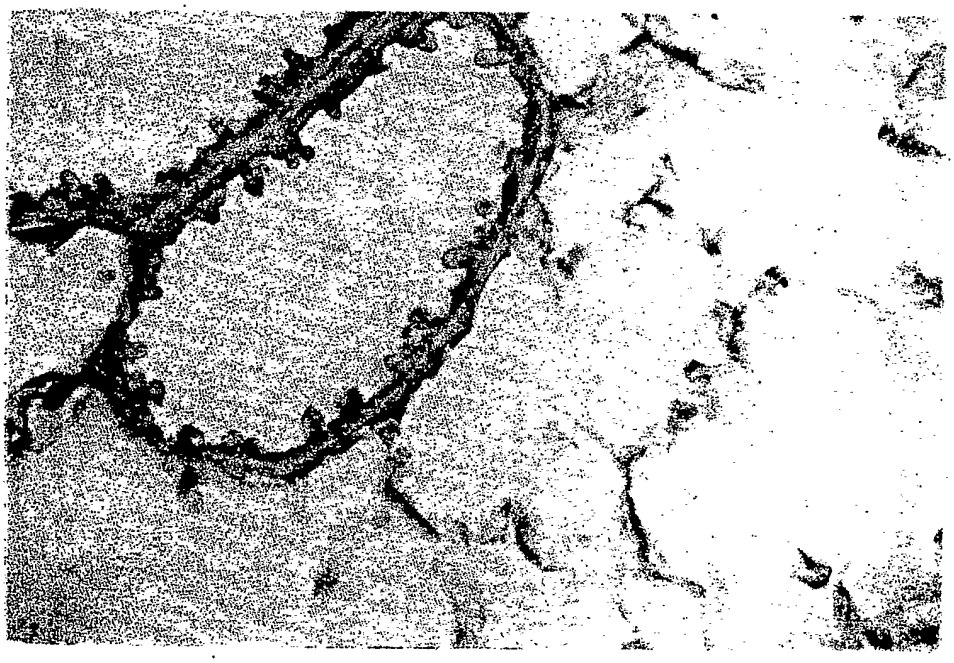


FIGURE 26C

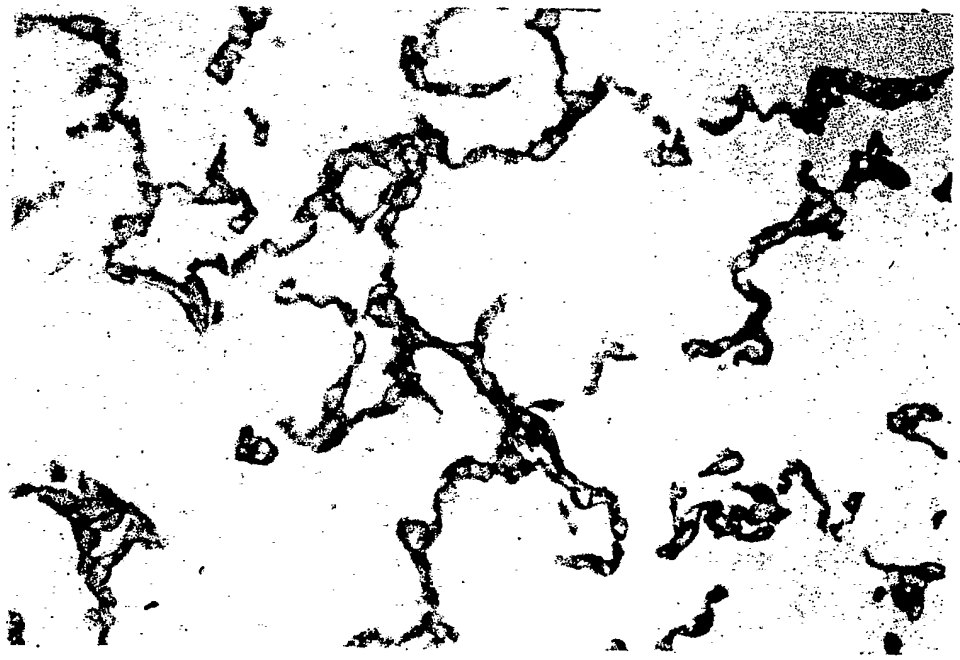


FIGURE 26D

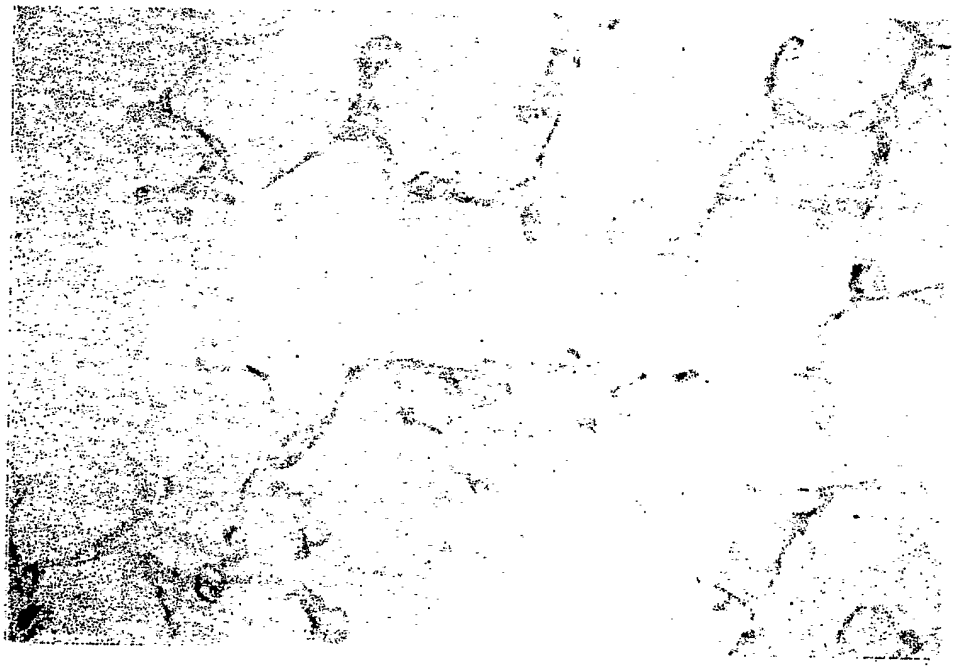


FIGURE 26E

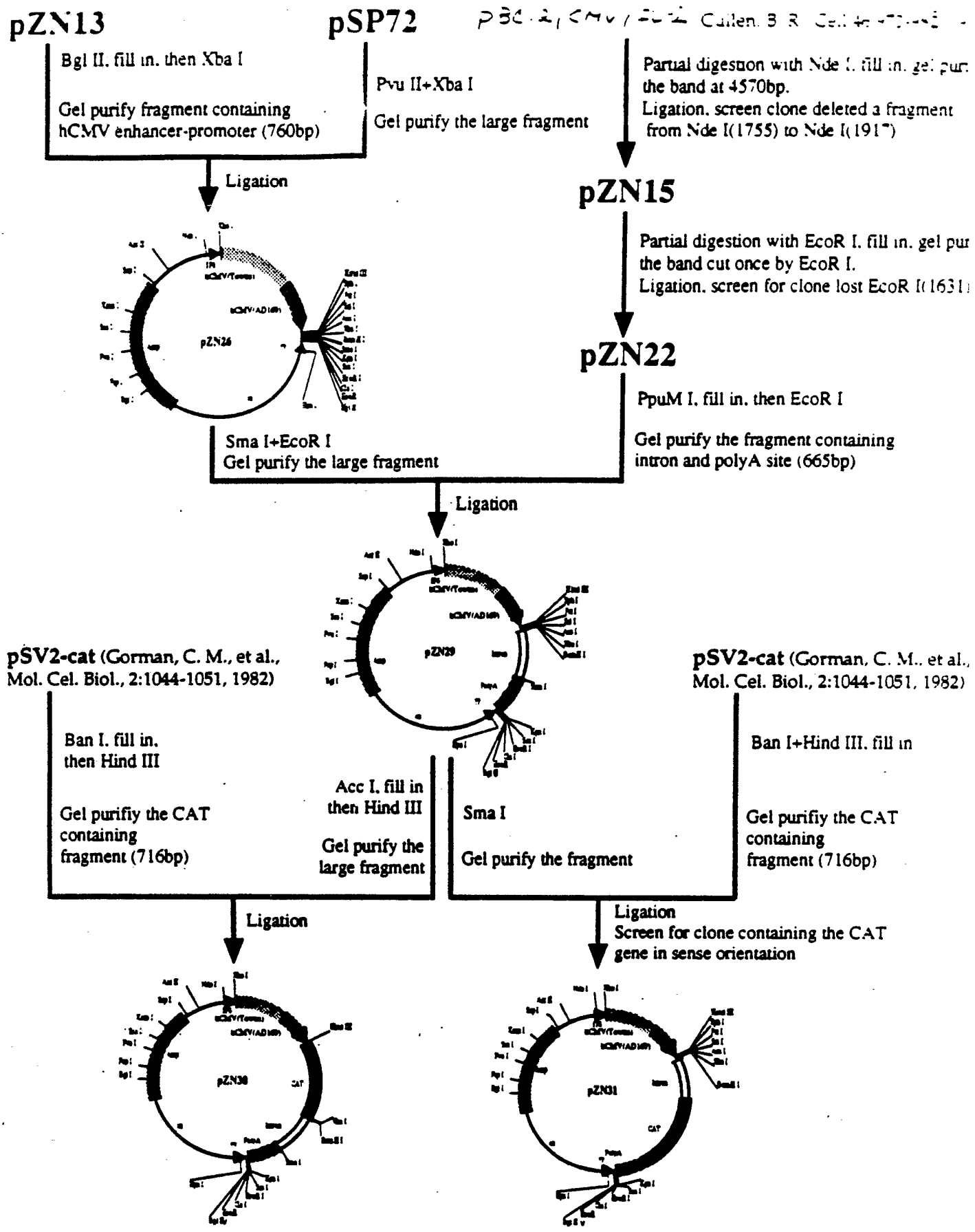

pZN13
pSP72
Bgl II. fill in. then Xba I
Gel purify fragment containing hCMV enhancer-promoter (760bp)
Pvu II+Xba I
Gel purify the large fragment
Ligation
pZN26
Partial digestion with Nde I. fill in. gel pur
the band at 4570bp.
Ligation. screen clone deleted a fragment
from Nde I(1755) to Nde I(191
pZN15
Partial digestion with EcoR I. fill in. gel pur
the band cut once by EcoR I.
Ligation. screen for clone lost EcoR I(1631
pZN22
PpuM I. fill in. then EcoR I
Gel purify the fragment containing
intron and polyA site (665bp)
Sma I+EcoR I
Gel purify the large fragment
Ligation
pZN29
pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)
Ban I. fill in.
then Hind III
Gel purifiy the CAT
containing
fragment (716bp)
Acc I. fill in
then Hind III
Gel purify the
large fragment
Sma I
Gel purify the fragment
pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 1982)
Ban I+Hind III. fill in
Gel purifiy the CAT
containing
fragment (716bp)
Ligation
Ligation
Screen for clone containing the CAT
gene in sense orientation
pZN30
pZN31

FIGURE 27A

pBC12/CMV/IL-2

(Cullen, B. R., Cell 46:973-982, 1986)

pZN29

Hind III+BamH I

Gel purify large fragment

Hind III+BamH I

Gel purify fragment containing IL-2 Gene (684bp)

Ligation

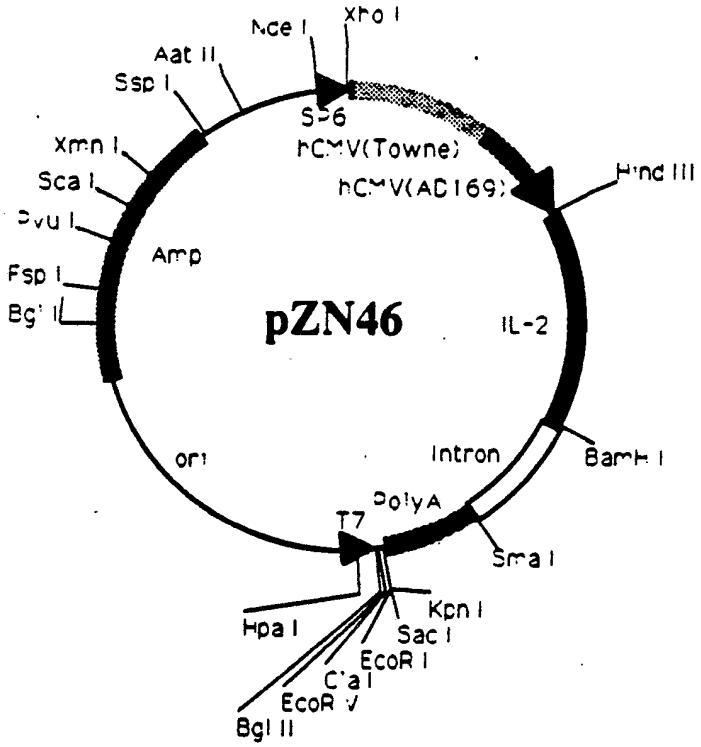


FIGURE 27B

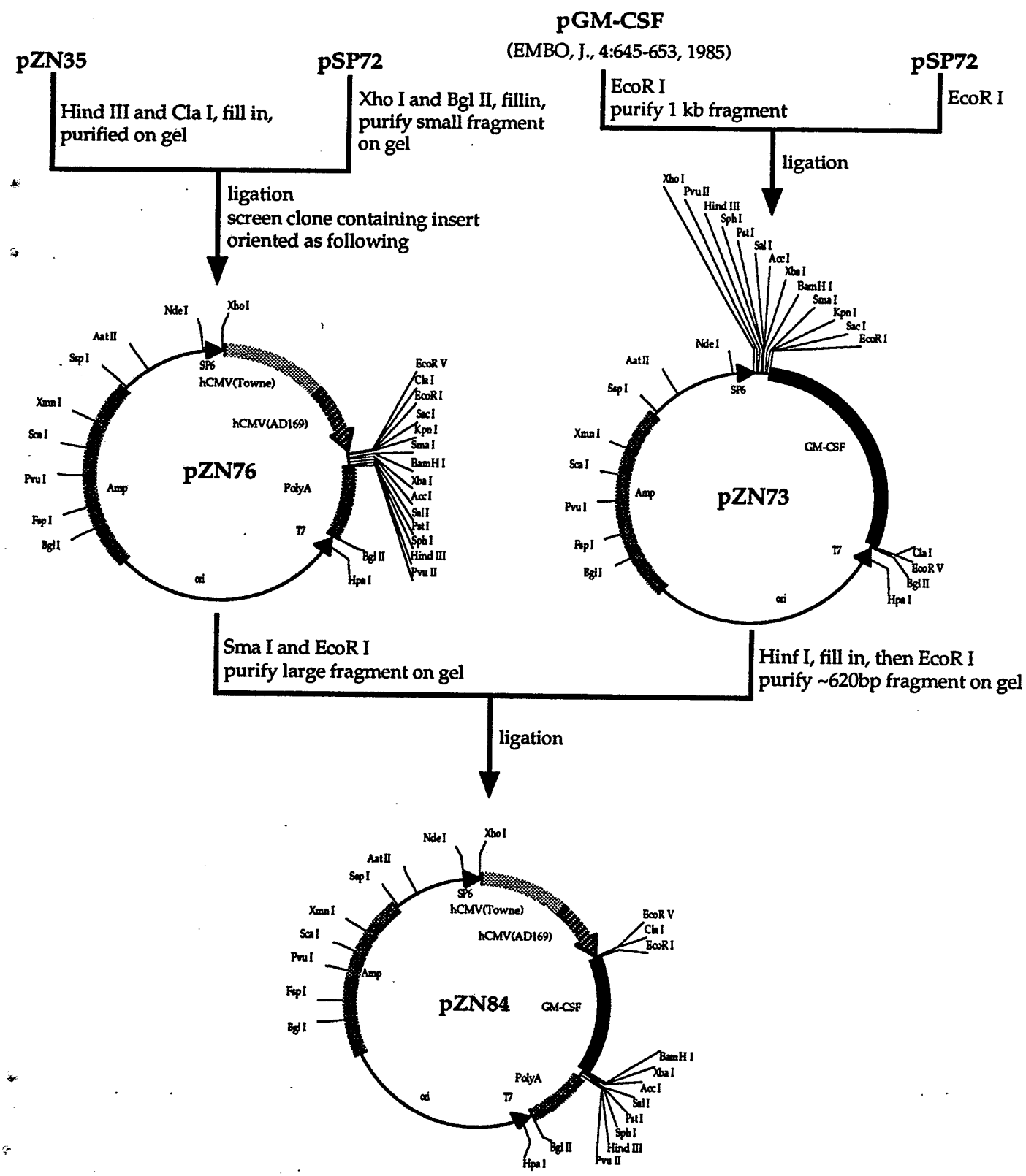

pGM-CSF
(EMBO, J., 4:645-653, 1985)
pZN35
pSP72
Hind III and Cla I, fill in, purified on gel
Xho I and Bgl II, fillin, purify small fragment on gel
ligation
screen clone containing insert oriented as following
pZN76
NdeI
XhoI
AatII
SspI
SP6
hCMV(Towne)
hCMV(AD169)
XmnI
ScaI
PvuI
Amp
PolyA
FspI
BglI
T7
ori
EcoR V
ClaI
EcoR I
SacI
KpnI
SmaI
BamH I
XbaI
AccI
SalI
PstI
SphI
Hind III
PvuII
BglII
HpaI
pSP72
EcoR I
EcoR I
purify 1 kb fragment
ligation
XhoI
PvuII
Hind III
SphI
PstI
SalI
AccI
XbaI
BamH I
SmaI
KpnI
SacI
EcoR I
NdeI
AatII
SspI
SP6
XmnI
ScaI
PvuI
FspI
BglI
GM-CSF
pZN73
Amp
T7
ClaI
EcoR V
BglII
HpaI
ori
Sma I and EcoR I
purify large fragment on gel
Hinf I, fill in, then EcoR I
purify ~620bp fragment on gel
ligation
pZN84
NdeI
XhoI
AatII
SspI
SP6
hCMV(Towne)
hCMV(AD169)
XmnI
ScaI
PvuI
Amp
FspI
BglI
GM-CSF
EcoR V
ClaI
EcoR I
BamH I
XbaI
AccI
SalI
PstI
SphI
Hind III
PvuII
PolyA
T7
ori
BglII
HpaI

FIGURE 28A

pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)

Xma I and Sal I,
gel purify the large fragment

pZN76

Xma I and Sal I

Ligation

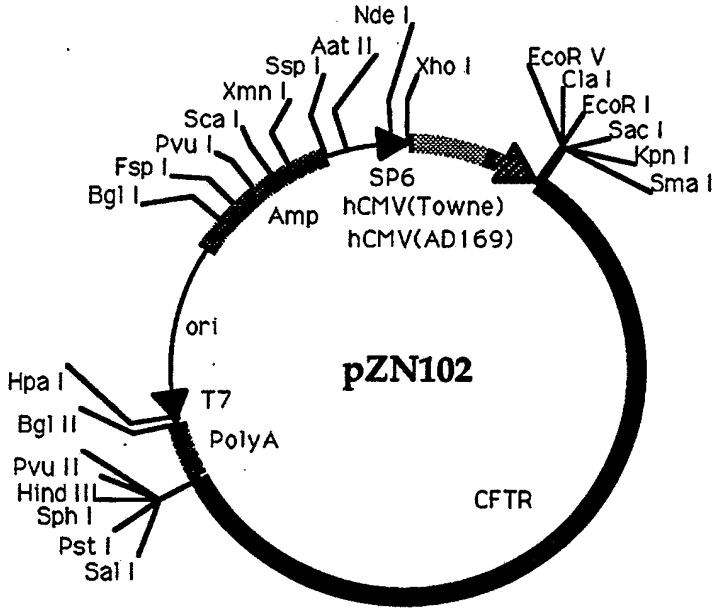


FIGURE 28B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/11004

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/88; A61K48/00; C12N15/12; A61K47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 89, no. 23, 1 December 1992, WASHINGTON US pages 11277 - 11281 STRIBLING, R. ET AL. 'Aerosol gene delivery in vivo'	1-28
P,Y	see the whole document ---	1-28
P,X	CELL. vol. 68, no. 1, 10 January 1992, CAMBRIDGE, NA US pages 143 - 155 ROSENFELD, M.A. ET AL. 'In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium'	1,2,5, 17,22, 24,25,28
P,Y	see the whole document ---	1-28
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 APRIL 1993	14. 05. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	NUCLEIC ACIDS RESEARCH. vol. 20, no. 12, June 1992, ARLINGTON, VIRGINIA US pages 3233 - 3240 YOSHIMURA, K. ET AL. 'Expression of the human cystic fibrosis transmembrane conductance regulator gene in the mouse lung after in vivo intratracheal plasmid-mediated gene transfer' cited in the application	1,2,5, 17,24, 25,28
P,Y	see the whole document	1-28
X	EP,A,0 446 017 (GENZYME CORPORATION) 11 September 1991 cited in the application see page 21, line 28 - page 22, line 2 see page 25, line 8 - line 15; claims 1-10,16,17	1,2,17, 24,25,28
Y	see the whole document	1-28
X	WO,A,9 102 796 (HSC RESEARCH DEVELOPMENT CORPORATION) 7 March 1991 see claims 90-92	1,7,8
P,X	WO,A,9 205 273 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 2 April 1992 see page 17, line 30 - page 18, line 2 see page 18, line 28 - line 35; claims	1-3,17, 21,28
P,Y	see the whole document	1-28
Y	US,A,5 049 386 (EPPSTEIN, D.A., FELGNER, P. L., THOMAS, R.G., JONES, G.H., RICHARD, B.) 17 September 1991 see column 9, line 9 see column 10, line 43 - line 68 see column 11, line 42 - line 53 see column 13, line 13 - line 17	1-28
Y	JOURNAL OF IMMUNOLOGY. vol. 140, no. 10, 15 May 1988, BALTIMORE US pages 3482 - 3488 DEBS, R.J. ET AL. 'Lung-specific delivery of cytokines induces sustained pulmonary and systemic immunomodulation in rats' see the whole document	1-28

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9211004
SA 69296

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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